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THE RESPONSES OF CATFISH MELANOPHORES TO ERGOTAMINE

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Some eight years ago Bacq (1933), on the basis of experimental evidence, reached the conclusion that ergotamine contracts denervated catfish melanophores and expands normally innervated ones. It is now known that the so-called contraction of catfish melanophores is the result of a neurohumor from the concentrating nerve-fibers, very likely adrenaline, and that their expansion is due to two agents, intermedine from the pituitary gland and a neurohumor, probably acetylcholine, from the dispersing nerve-fibers (Chang, Hsieh, and Lu, 1939; Parker, 1940). In consequence of these new discoveries it seemed desirable to repeat Bacq's experiments with the view of bringing his rather remarkable results into relation with this newly acquired information.

The ergotamine tartrate used by Bacq is fortunately still to be had in the American market. It is dispensed in 1 cc. glass ampules under the name of gynergen (Sandoz Chemical Works) and in this form it is extremely convenient for experiments on fishes. Three sets of catfishes (*Ameiurus nebulosus*) were prepared for these tests,—pale, intermediate, and dark. The pale fishes, three in number in the initial set, were kept in white-walled, illuminated vessels. Two caudal bands were cut in each fish. By the end of six days these fishes were very pale and their caudal bands were almost fully blanched. The axis and tip of each band, however, were noticeably dark as observed and figured by Bacq (1933). The three fishes of intermediate tint were kept in a gray, illuminated vessel. Their caudal bands after six days were very slightly darker than the rest of their darkish tails. The three fishes of the dark set were rendered very dark, coal-black, by complete blinding. It is well known that catfishes assume this intense shade on double enucleation. Notwithstanding the great depth of tint thus produced, the caudal bands in these fishes were a shade darker than the rest of their very dark tails.

Six days after the cutting of the caudal bands in the pale fishes these bands were recut a little distal to the original incision. Since the part of the band distal to the new cut did not change in tint as a result of the recutting, it was concluded that so far as color changes were concerned the nerves of such bands had degenerated. As the three fishes in any given set, pale, intermediate, or dark, were very similar in color, one in each set was reserved as a control and the other two were subjected to tests. Two injections each of 0.25 cc. of gynergen separated by an interval of about a quarter of an hour yielded the best results. These injections were at times supplemented by a third. Two injections of 0.5 cc. of gynergen with an interval of fifteen minutes between them gave more vigorous responses than the weaker injections, but they were usually followed some hours later by the death of the fish. I was unable to obtain unquestionable responses with only a single injection of 0.25 cc. of gynergen as reported by Bacq. The catfishes used by me weighed each about 50 grams. Bacq makes no statement as to the weight of his specimens. Possibly he had smaller individuals than I had and therefore obtained satisfactory responses with less ergotamine. In my procedure any given catfish must have received into its body from the two injections ordinarily given a total amount of 0.25 mg. of ergotamine tartrate judged from the formula published by the Sandoz Chemical Works for their preparation of gynergen.

Bacq's tests, which were carried out only on pale catfishes, consisted in injecting into such a fish with a blanched caudal band 0.25 cc. of gynergen whereupon the fish as a whole became dark, but the band remained pale or even took on a somewhat lighter tint. My repetition of such a test gave almost identical results. When a pale fish with two blanched caudal bands was injected with the usual two doses of gynergen, 0.25 cc. each, with an intervening quarter of an hour, the fish began to darken noticeably in about half an hour after the first injection and in an hour to an hour and a half it had reached a full intermediate tint, its maximum color change under the circumstances. As the tail darkened the bands appeared to become paler as noted by Bacq, but whether this was an actual blanching or a contrast phenomenon could not be settled except by close scrutiny. When the bands on the tail of an injected fish were closely compared with those on the uninjected pale control, the two sets of bands were found to be in very close agreement. This was particularly well seen under a low power of the microscope. In both sets of bands the pigment masses in the macromelanophores were rounded bodies with short, blunt protuberances on their sides marking the roots of the pigmented processes of the dispersed stage. The pigment masses in the injected fishes appeared to be in no sense less dispersed than those

in the control fish, and yet when the bands were inspected by the unaided eye those in the dark fishes appeared to be paler than those in the pale control. In my opinion this apparent difference is purely an illusion due to contrast. The dark surroundings of the pale bands in the injected fishes made these bands appear paler than the pale bands in the pale control. I therefore conclude that, contrary to Bacq's view, ergotamine has no effect on denervated melanophores with concentrated pigment. This agent, however, does induce pigment dispersion in innervated color cells, as stated by Bacq.

In one other respect my observations do not agree with those of Bacq. In the majority of caudal bands that have been blanched in pale fishes for some six days the axes and tips of these bands, as already stated, are slightly dark. This feature was described and figured by Bacq, who noted further that when catfishes showing these peculiarities were injected with ergotamine the pale bands not only became paler but their dark axes and tips also blanched. In my experience such was not the case. After full doses of ergotamine had been allowed to act on the two pale catfishes tested by me, the dark axes and tips in their caudal bands were as visible after the injection as they had been before it or as they were in the control.

In making these several comparisons the individual catfishes in the course of inspection were necessarily much handled. As is well known, this treatment induces such fishes to darken temporarily and it might be supposed that this darkening could in some way have influenced the results just described. But both the control fish and the two injected individuals were handled to about the same degree and therefore should have shown the same amount of change as a result of this treatment. Moreover, it has been demonstrated in a recent paper (Parker, 1940) that the darkening already alluded to is a response mediated by the dispersing nerves. Consequently it ought to play no part in the activities of a denervated area such as a caudal band. There is therefore no reason to suppose that the ordinary darkening of catfishes from handling could have had any influence on the results herein recorded.

The tests on the three pale catfishes just described were repeated on two other sets of pale individuals, one of two fishes and the other of three. In both these sets the pale bands of the injected fishes showed no more change in tint than did those of the first set and their bodies in general darkened to intermediate. This agreement in three sets of results justifies the conclusion that, as Bacq maintained, ergotamine excites innervated melanophores in catfishes to disperse their pigment to a point where the fish attains an intermediate tint. It shows further that, contrary to Bacq's opinion, this agent does not induce a concentration of

pigment in denervated melanophores whereby caudal bands in pale fishes would become still paler. Ergotamine apparently has no influence whatever on denervated melanophores with concentrated pigment. It does induce pigment dispersion in innervated melanophores.

When catfishes with caudal bands cut in their tails are kept for some six days in a gray, illuminated vessel they take on, as already stated, an intermediate tint in which the bands are as a rule slightly darker than the rest of the fish. If these fishes now receive the usual injections of ergotamine, two doses of gynergen, 0.25 cc. each, separated by a quarter of an hour, they will either show no noticeable change in tint at all or darken very slightly. This rule held for all three sets of catfishes tested, including a total of nine individuals. In no instance was there any evidence of the blanching of the denervated bands, but, contrary to what might have been expected from Bacq's statements, these bands remained usually a little darker than the rest of the tail. Bacq apparently never tested fishes of intermediate tint with ergotamine. Had he done so, he surely would have observed that when the ergotamine excited any change at all it was a very mild darkening in the region of the innervated color cells and not only no blanching but no change of color whatever in that of the denervated cells.

What has been stated for catfishes of an intermediate tint may be affirmed in general for those that are fully dark. As noted previously, the caudal bands in such fishes are as a rule very slightly darker than the rest of the tails in these individuals. After the usual injections of ergotamine these color conditions either showed no change or the fish as a whole darkened very slightly. In two instances this general darkening was sufficient to make the tail slightly darker than the bands. As comparisons with the control fish showed, this was a real darkening of the tail and not a slight blanching of the band. Hence we are justified in concluding, as in the instance of the intermediate fishes, that ergotamine induces pigment dispersion in innervated melanophores but has no effect on denervated ones.

From these several tests on pale, intermediate, and dark catfishes it seems fair to conclude that ergotamine acts only on innervated melanophores by inducing them to disperse their pigment and has no effect whatever on denervated melanophores. The assumed blanching of these color cells by this agent, as described by Bacq, is purely illusory. So far as the end result is concerned, ergotamine is like intermedine or acetylcholine in that it causes catfishes to darken. But it does not act in the same way as these two neurohumors do. They act directly on the melanophores (Parker, 1941), for they will induce denervated caudal bands to darken. Ergotamine acts on melanophores indirectly, that is through

nerves, in that it excites at some central nervous station the dispersing nervous elements which in turn excite the appropriate nervous terminals to discharge acetylcholine. This neurohumor causes the melanophores to disperse their pigment whereby the fish darkens. Ergotamine is a good example of an indirect melanophoric agent as contrast with direct ones such as intermedine, acetylcholine, and adrenaline. These will activate denervated melanophores in caudal band. Ergotamine is incapable of this activity.

Summary

1. Ergotamine acts on only innervated melanophores by inducing them to disperse their pigment. It is without effect on denervated melanophores either with dispersed or with concentrated pigment.

2. It acts on innervated melanophores only indirectly, that is, through their nerves. These are excited by ergotamine centrally to produce at their melanophore terminals acetylcholine which causes the color cells to disperse their pigment.

3. Ergotamine is a good example of an *indirect* excitant of melanophores as contrasted with *direct* excitants such as intermedine, acetylcholine, and adrenaline, all of which act directly on these color cells.

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SEXUAL PHASES IN WOOD-BORING MOLLUSKS

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It has been demonstrated previously that the well-known shipworm *Teredo navalis* is typically protandric, nearly all individuals functioning as males when young and later changing to the female phase (Coe, 1936; Grave and Smith, 1936). Under favorable conditions the female phase may be followed by an additional sequence of male and female phases.

In no other species of the wood-boring mollusks (Teredinidae) is the sexual sequence fully known, although Yonge (1926) recognized protandry in *Teredo norvegica* and Siegerfoos (1908) concluded that it was present in *"Xylotrya gouldi"* = *Bankia fimbriata* (Jeffries).

During the past few years the writer has had the opportunity of investigating the biology of three species, *Teredo navalis, T. diegensis* and *Bankia setacea,* with particular reference to the development of the gonads and the sequence of the sexual phases. The results of this study may be briefly summarized and compared with previous reports on the sexuality of the shipworms.

Sexual Phases of Teredo navalis

This widely distributed species occurs on both the Atlantic and Pacific coasts of the United States and has been particularly destructive in past years in San Francisco Bay. Individuals in the female phase are larviparous, the fertilized eggs developing through about half the larval period in the maternal gill chambers. A free-swimming period follows the discharge of the larvae into the water. After settling upon a piece of wood, transformation to the adult condition takes place and boring into the wood begins (Kofoid and Miller, 1927; Grave, 1928).

The primary male phase becomes functional within four to six weeks after the completion of the free-swimming larval condition in the warmer season of the year or in warm localities, but may be delayed for six months or more under colder conditions. The body is then only 20 to 30 mm. in length and about 2 mm. in diameter. The female phase may begin at the age of eight to ten weeks.

[1] Contributions from the Scripps Institution of Oceanography, No. 145.

Growth is rapid under favorable conditions. At the end of one year the body may have attained a length of 10 to 40 cm. and a diameter of 4 to 9 mm. In the meantime the individual has normally transformed to the female phase and has produced perhaps 1,000 000 young. A second sequence of male and female phases may have occurred.

Because of the long breeding season and the sequence of sexual phases, the proportion of the two sexes in each piece of wood will obviously depend upon the ages represented in the colony. In a recently attacked timber nearly every individual will be in the male phase. A few weeks later, after the sexual transformation has occurred, 60 to 90 per cent of the original colony will be functioning as females.

But in the meantime there may have been daily additions of recently arrived young from other pieces of wood, resulting in a continuing supply of male-phase individuals. These, together with the few so-called true males, and a small proportion of second male-phase individuals, are then available for the fertilization of the eggs produced by such individuals as are at that time functioning as females. Because of this overlapping of sexual phases all the older colonies are at all times represented by both sexual phases in varying proportions. Most of the smaller and consequently younger individuals will be functioning as males, while most of the larger, older individuals are in the female phase.

A rhythmical sequence of four sexual phases may be considered to represent the normal life cycle but this is seldom realized because of an earlier death due to parasites or to the exhaustion of the wood supply or to other unfavorable environmental conditions. Most individuals die after only two of these phases have been completed and many others survive only the primary male phase.

There are some variations in this sequence, however, because a second female phase may sometimes follow the first without an intervening male phase. Other individuals, known as true males, retain the male phase long after their contemporaries have changed to the female condition, and this may sometimes mean throughout their entire lifetimes.

Sexual Phases in Teredo diegensis

This species, like *T. navalis,* is protandric and larviparous, but the two species differ considerably both in the conditions of sexuality and in larval development.

T. diegensis Bartsch occurs abundantly and causes considerable damage along the coast of Southern California, and has been reported as far north as San Francisco (Kofoid and Miller, 1927). It is also found at the Hawaiian Islands. On the coast of Southern California this spe-

cies breeds through all except the two or three cooler months of the year and through the entire year when the winter is warmer than usual. At the lower temperatures the larvae may remain within the maternal gill chambers for several months before they are discharged.

Ovulation occurs at intervals of a few weeks, the later broods of larvae often becoming established in the gills before an earlier brood has left. This condition occurs throughout the year. With the exception of young individuals and a few "true males" a sexually mature individual without a brood of larvae is seldom found.

Large individuals, 120 mm. or more in length, may have more than 1000 larvae in the gill chambers, while dwarfs may have less than 100. The bivalve larvae reach a shell length of 0.35 to 0.38 mm. before leaving the gills. In this species, as in *T. pedicellata* (Roch, 1940), the larvae remain within the gill chambers until nearly ready for metamorphosis. The free-swimming stage consequently lasts but a few hours if wood is available for attachment. The total period of larval development is about four weeks and is therefore of about the same length as in *T. navalis* (Coe, 1933*a*). In this latter species, however, only about two weeks of this time are spent in the maternal gill chambers, followed by a free-swimming period of about the same duration.

Shortly after being set free in the water the larvae of *T. diegensis* attach themselves to any available piece of wood but do not immediately penetrate the surface. Some of them may remain two weeks or more on the surface of the wood before beginning to bore. Their stomach contents show that minute particles of organic food materials are ingested in the meantime. Because there is no necessity for feeding during the brief free-swimming stage, this species may be reared from generation to generation in the aquarium. By supplying a fresh piece of wood occasionally the stock may be continued for at least several years. The aquarium water evidently contains sufficient materials to supplement the wood as sources of nourishment. After penetrating the wood the young teredos grow rapidly and reach the primary male phase within four to five weeks. The body is then about 8 to 12 mm. in length.

The primary gonad is composed of branching follicles filled with large, vacuolated cells and having a few proliferating germinal cells scattered along the walls of the follicles as shown for *Bankia setacea* (Fig. 1, *A*). This condition is closely similar to that described by Coe and Turner (1938) for the developing gonads of *Mya*. As the germinal cells increase in number they become differentiated into the two sexual types of gonia and then further differentiated into ovocytes and spermatocytes.

By their rapid proliferation and differentiation the spermatogenic cells encroach upon the spaces occupied by the vacuolated follicle cells and eventually fill the entire lumen of the follicle (Fig. 1, *B*). In some individuals the follicle cells contain numerous fragmenting and degenerating nuclei, representing a kind of atypical spermatogenesis, as described by Coe and Turner (1938) for *Mya*.

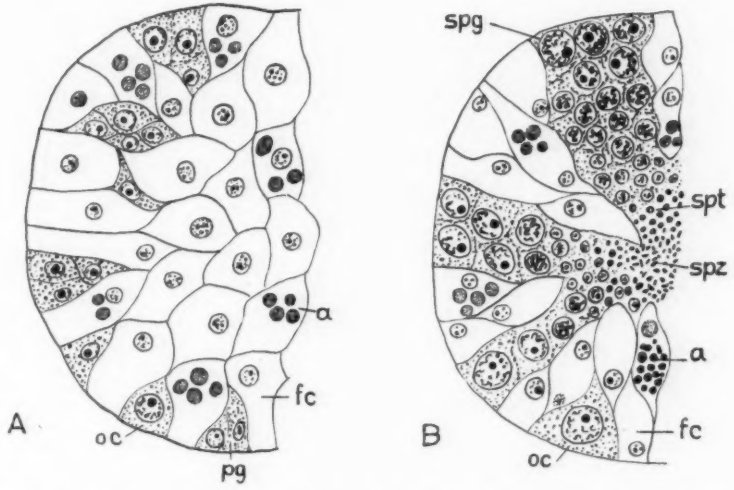

FIG. 1. *Bankia setacea*. Development of the primary ambisexual gonad. *A*, portion of section of young follicle, showing large vacuolated follicle cells (*fc*), with a few primary gonia (*pg*) and a single young ovocyte (*oc*) peripherally; several follicle cells contain atypical, degenerate nuclei (*a*), derived from original primary gonia. *B*, portion of follicle in early male phase, with a few remaining vacuolated follicle cells (*fc*) and various stages of spermatogenesis; *a*, atypical degenerate nuclei; *oc*, ovocyte; *spg*, spermatogonia; *spt*, spermatids; *spz*, spermatozoa.

There is considerable variation in the number and size of the ovocytes which are always present on the walls of the follicles during spermatogenesis. Some individuals, corresponding with the so-called true males of *T. navalis*, have only a few small ovocytes in each of the follicles (Fig. 3, *A*), while others show a preponderance of ovocytes in some or all of the follicles before the spermatozoa are fully ripe (Fig. 2).

Occasionally all the spermatozoa are discharged before ovulation occurs, resulting in a distinctly female phase (Fig. 3, *B*), but more frequently a functionally hermaphroditic condition is found. Both spermatozoa and ova may ripen at the same time and evidently both

may be discharged simultaneously. Under experimental conditions self-fertilization occurs readily; this is followed by the formation of the polar bodies and cleavage, but only as far as the blastula and gastrula stages. For the normal processes of larval development the environmental conditions peculiar to the maternal gill chambers appear to be necessary.

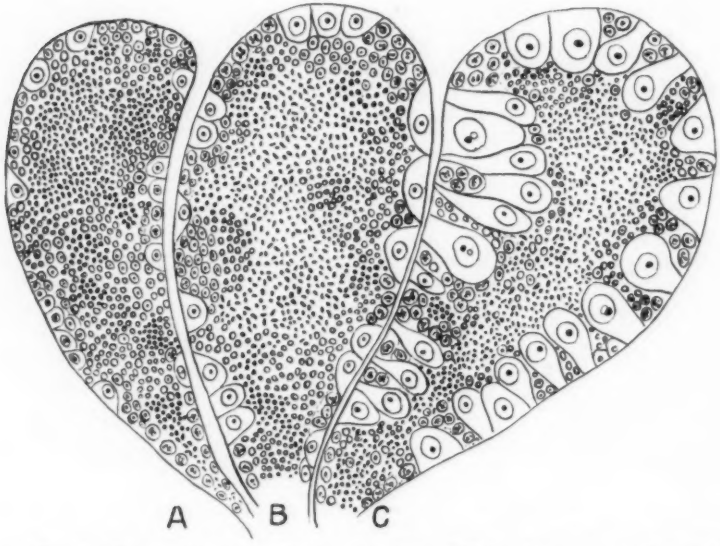

FIG. 2. *Teredo diegensis.* Sections of three follicles from gonad of second male-phase individual which had branchial brood pouches distended with larvae. *A*, immature follicle, principally in male phase, with relatively few ripe spermatozoa and with only small ovocytes in basal layer of germinal cells. *B*, more nearly mature follicle in male phase, distended with spermatogenic cells and many ripe spermatozoa; numerous half-grown ovocytes in basal layer of germinal cells. *C*, ripe follicle in hermaphroditic male phase; lumen filled with ripe spermatozoa and with nearly mature ovocytes densely crowded on periphery.

During some seasons this species becomes particularly injurious by boring in mooring ropes. A similar habit has been reported for *T. navalis* (Coe, 1933). Under such conditions only dwarf individuals are produced but many of these are nevertheless capable of forming a small number of ripe gametes. A single change of sexual phase, from male to female, may occur, although many individuals are killed by the disintegration of the rope before even the primary male phase is completed.

Sexual Phases of Bankia setacea

This species differs from *Teredo diegensis*, with which it is often associated in the cooler waters on the coast of southern California, in being oviparous rather than larviparous. On reaching the female phase vast numbers of minute ova are produced and these are discharged directly into the water. Fertilization of these ova by sperm of other individuals takes place in the water. Then follows a free-swimming larval period of perhaps four weeks before the larva is ready to settle on a piece of wood and transform to the adult condition.

It is evident that all individuals pass through a functional male phase a few weeks after entering the wood. The two types of males are more

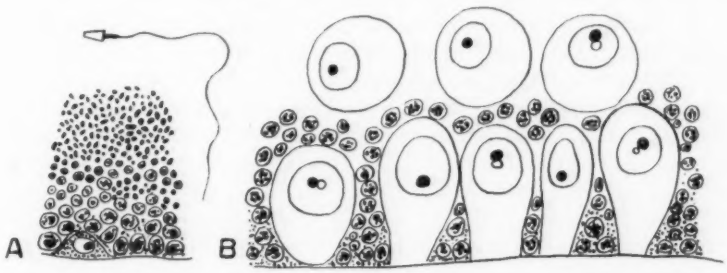

Fig. 3. *Teredo diegensis*. *A*, portion of gonad of young individual in "true male" phase, showing spermatogenesis and a single, more highly enlarged spermatozoön; a single ovocyte is shown at the base of the spermatogenic cells. *B*, portion of gonad in female phase, with ripe ova; those ova still attached to wall of follicle are surrounded by follicle cells with undifferentiated gonia basally.

easily distinguished than in either *Teredo navalis* or *T. diegensis*. About half of the young, male-phase individuals are apparently hermaphroditic; these complete spermatogenesis early and then change to the female phase. These are evidently genetically protandric females, while an approximately equal number retain the primary male phase much longer. The latter presumably represent the "true males" of other species (Coe, 1933*a*, 1936). Fully adult individuals, measuring 20 to 50 cm. in length, usually have the appearance of being either males or females, with little indication of ambisexuality. Only occasionally do their gonads reveal distinctly their essentially hermaphroditic nature. Careful examination of the gonad in full spermatogenesis, however, usually reveals a few ovocytes on the walls of the follicles and these may be considered as an indication that a change of sexuality may later occur. In the female phase likewise indifferent gonia show the potentiality of a sex change.

The sexual conditions in this species are therefore similar to those of the oyster *Ostrea virginica* (Coe, 1938), which is seasonally of separate sexes but in which the sexual phase of any season cannot be predicted from the sexual condition of the preceding season. In *Bankia*, however, many of the females experience but a single change of sex, from male to female, while the true males may retain the male phase throughout life.

On the coast of southern California some individuals of *B. setacea* are found in spawning condition throughout the year. The majority of individuals, however, spawn only during the autumn and spring months, with resting periods in the winter and summer. Consequently wooden blocks and timbers become much more quickly and more heavily infested in the spring and autumn than at other times of the year.

Johnson and Miller (1935) found that settlement of this species in Puget Sound occurred principally from October to December and less abundantly from March to September. Kofoid and Miller (1927) also observed that in San Francisco Bay settlement of this species was confined to the cooler months of the year.

When removed from the body the eggs of *Bankia* develop rapidly to the free-swimming larval condition after artificial fertilization by sperm from another individual. The eggs of juvenile protandric females are sometimes capable of self-fertilization.

Conclusions

It is evident that the sexuality of these three species of pelecypods represents a graded series of ambisexual or hermaphroditic conditions intermediate between such dioecious forms as *Mya* or *Mytilus*, which are almost strictly of separate sexes, and those that are uniformly monoecious, as the larviparous oysters, *Ostrea edulis* or *O. lurida*. In all the dioecious pelecypods of which the sexuality has been extensively investigated hermaphroditism is found occasionally and this may include a large or only a small portion of the gonad.

Even the monoecious species usually have some dioecious tendencies, with some individuals ("true males") showing a preponderance of masculine characteristics, while others are more nearly feminine. A sequence of functional male and female phases is of common occurrence and in the case of long-lived species this may constitute an alternating rhythm. The wood-boring mollusks are of this type.

Protandry is characteristic of many species. This represents a juvenile type of sexuality and often occurs when the individual is very young and when the body has reached but a small fraction of its definitive size.

As mentioned above for *Bankia* and as Loosanoff (1937) found in *Venus*, the genetic females pass through a juvenile male phase before adult sexuality is realized. This has been reported for other species. In the oviparous oyster *Ostrea virginica* the proportion of the genetic females which pass through a functional male phase during their first breeding season depends both upon the particular local race concerned and upon the environmental conditions. This juvenile male phase is more frequently aborted or omitted under conditions favorable to rapid growth, thereby increasing the proportion of juvenile females. This species also shows a rhythmical tendency toward seasonal change of sex in later life (Coe, 1938).

In all the examples mentioned and in many others belonging to the various classes of mollusks, the sex-differentiating mechanism is so delicately balanced between the two sexual tendencies that relatively slight differences in environmental conditions may be potent in determining which of the two contrasted aspects of sexuality shall be realized. In some the entire population functions as male when young and as female when fully adult. An intervening functionally hermaphroditic phase may occur.

Summary

1. The three species of wood-boring mollusks *Teredo navalis*, *T. diegensis* and *Bankia setacea*, are all protandric, with a strong tendency toward rhythmical changes of functional male and female phases.

2. Each species differs as to the degree of ambisexuality characteristic of the primary male phase and of the subsequent sexual phases.

3. The primary gonad in all three species develops from branching follicles filled with large vacuolated follicle cells and having the primary gonia scattered along walls of the follicles.

4. In each of these species the gonads of young animals indicate that there are two types of primary male-phase individuals: (1) ambisexual males or protandric females, characterized by many ovocytes on the walls of the spermatic follicles, and (2) true males with few ovocytes. In those of the former type the male phase is of short duration, while true males retain the male phase longer or in some cases indefinitely.

5. In *T. navalis* the first female phase does not usually become functional until nearly all the sperm of the primary male phase have been discharged. Functional hermaphroditism is not usual, although the gonad is histologically ambisexual during the change of sexual phase in both directions.

6. In *T. diegensis*, on the other hand, functional hermaphroditism is of usual occurrence and the sexual phases are not sharply demarcated.

7. In *B. setacea* functional hermaphroditism occurs only occasionally in the primary male phase; the subsequent sexual phases are clearly differentiated, often with a resting stage intervening between two sexual phases. The sexual phases are of the alternative type in that any sexual phase, after the first, may be followed by either a male or female phase if the length of life suffices. The relatively short life of many individuals, however, allows but a single change of sex, from male to female, in the genetic females, and none at all in true males.

8. In all of the three species the eggs begin development after artificial fertilization. In *Bankia* the larvae may be reared to the free-swimming veliger stage, but in the other two species the larval stages require the peculiar environmental conditions of the maternal gill chambers. Under experimental conditions self-fertilization and apparently normal cleavage occurs readily in the two species of *Teredo* and occasionally in *Bankia*.

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REGENERATION OF COENOSARC FRAGMENTS REMOVED FROM THE STEM OF TUBULARIA CROCEA

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The rôle of environmental factors in the regeneration of hydroids has been studied extensively, the evidence all pointing to the extreme lability of hydroid systems. The literature on this subject has recently been reviewed by Barth (1940*b*). Morgan (1903) found that, when the cut end of a *Tubularia* stem was placed in sand, regeneration was inhibited at that end. Regeneration is inhibited also when the sectioned ends of a *Tubularia* stem are ligated. The reason for this inhibition was made clear by experiments in which *Tubularia* stems were exposed to a differential of oxygen in the sea water. Barth (1938*a*) was able to reverse the normal polarity by placing the distal end of the stem in a glass capillary, and he attributed this reversal to a lack of oxygen at the covered end. Miller (1937) reversed the polarity of *Tubularia* stems by exposure of the proximal end to a higher oxygen tension. Further, the rate of regeneration was shown to be dependent upon the oxygen tension (Barth, 1938*a*). All of these experiments have been interpreted as meaning that regeneration in *Tubularia* is dependent upon the availability of oxygen (Barth, 1940*b*).

Experiments designed to determine the origin of polarity in regenerating *Tubularia* stems are complicated by the presence in these stems of an already existing polarity. This polarity is evidenced by a gradient in the rate of regeneration and a gradient of oxygen consumption in the stems (Barth 1938*b*, 1940*a*), and by the dominance of distal over proximal levels (Barth, 1938*b*). Direct exposure of the coenosarc to sea water provides a sufficient stimulus for regeneration (Zwilling, 1939), and since the process of regeneration involves reorganization of cells, it was indicated that exposure of the entire coenosarc surface to sea water might result in sufficient reorganization to obliterate the existing gradients in the stem. The coenosarc fragments could then be subjected to carefully controlled environmental differentials in an attempt to determine the rôle of the environment in regeneration.

The experiments were therefore designed to ascertain: (1) the nature of the structural changes which occur during the development of coeno-

sarc fragments; (2) whether there is a gradient of oxygen consumption in coenosarc taken from different levels of the stem; (3) the polarity exhibited by coenosarc fragments during regeneration.

Methods

During June and July the experiments were performed on *Tubularia crocea* collected from the wharf piles at the Marine Biological Laboratory. From September through December colonies were collected from floats in the Far Rockaway channel in New York City. Uniform, clean stems 5 to 8 cm. in length were chosen for the experiments. Segments 10 mm. long were used, the hydranth plus the first 5 mm. being discarded. The cuts were made with iridectomy scissors. Holding the perisarc at one end of the stem segment with a jeweler's forceps, a needle was passed gently over the perisarc, and the coenosarc expressed at the opposite end. During the summer most satisfactory survival and regeneration were obtained when the coenosarc fragments were kept in running sea water which had been filtered through absorbent cotton. The coenosarc fragments were placed on agar (2 per cent agar in sea water) in Syracuse dishes, and the latter transferred to a large glass aquarium through which the filtered sea water constantly flowed. The fragments were kept one-half inch from the surface of the water by elevating the Syracuse dishes in the aquarium. This was done to insure the availability of oxygen. The coenosarc fragments were moved around in the dish every few hours to insure uniform healing. Twenty-four hours after removal, they were transferred to Syracuse dishes which contained no agar. The coenosarc fragments removed from stems collected at Far Rockaway during the fall and winter were very hardy, satisfactory viability and regeneration being obtained using filtered sea water. Agar and continuous circulation of the sea water were not necessary. The operations and observations

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Explanation of Plate I [1]

Figs. 1–3, and 8–11 (× 15); Figs. 4 and 6 (× 160); Figs. 5 and 7 (× 950).

1. Coenosarc fragment (above) and empty perisarc (below) immediately after expression of the coenosarc.

2 and 3. Coenosarc fragment two hours (Fig. 2) and twenty-four hours (Fig. 3) after removal from the perisarc.

4 and 5. Section of coenosarc fragment immediately after removal.

6 and 7. Section of coenosarc fragment two hours after removal.

8–11. The types of regenerants obtained from expressed coenosarc fragments. Unipolar (Fig. 8); bipolar (Fig. 9); bipolar-unipolar (Fig. 10); multipolar (Fig. 11).

[1] The authors wish to thank Mr. Jack Godrich for his assistance in the preparation of the photomicrographs.

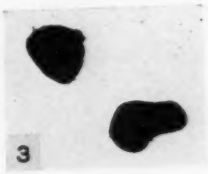

PLATE I

were made with the aid of a binocular microscope. The coenosarc fragments were fixed in Bouin's picro-formol-acetic fixative. They were sectioned at five microns, and stained with Delafield's haematoxylin.

The Development of Coenosarc Fragments

When coenosarc is removed from the perisarc, the tissues undergo a series of structural changes resulting, finally, in regeneration of hydranths. For convenience of description, the process may be divided into six stages based on characteristic morphological relationships.

Stage 1. The coenosarc has just been removed from the perisarc. There has been some morphological disturbance due to the mechanics of the operation. Plate I, Fig. 1, shows the condition of the coenosarc, above, and the empty perisarc, below. When examined histologically (Pl. I, 4), it may be noted that the ectodermal and endodermal layers are well defined, although the coelenteron has been obliterated. The nuclei of the ectodermal cells are centrally located and there is no trace of perisarc present (Pl. I, 5).

Stage 2. Two hours after removal the fragment has begun to contract along the original distal-proximal axis (Pl. I, 2), and the interior has become somewhat vacuolated (Pl. I, 6). The ectoderm is still well defined, but the nuclei of the ectodermal cells are located peripherally and the cells are somewhat elongated and swollen (Pl. I, 7). No perisarc is present. The atypical appearance of the ectodermal cells may be an indication of cellular degeneration, which is followed by a sloughing off of the original ectodermal cells. This loss of cells from the coenosarc may be observed, with the aid of a binocular microscope, from the time the perisarc is removed until a new perisarc is formed.

Stage 3. Twenty-four hours after removal, the coenosarc has undergone further contraction and is now somewhat spherical (Pl. I, 3). The outer layer is not well defined, and the inside of the spherical mass consists of numerous, closely packed cells (Pl. II, 12, 13). Traces of new perisarc may be noted around the periphery of the tissue mass (Pl. II, 13).

Stage 4. Thirty-six hours after removal, the center of the tissue

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Explanation of Plate II

Figs. 12, 14, and 16 (× 160); Figs. 13, 15, and 17 (× 950).

12 and 13. Section of coenosarc twenty-four hours after removal from the perisarc.

14 and 15. Section of coenosarc thirty-six hours after expression.

16 and 17. Section of coenosarc sixty hours after expression.

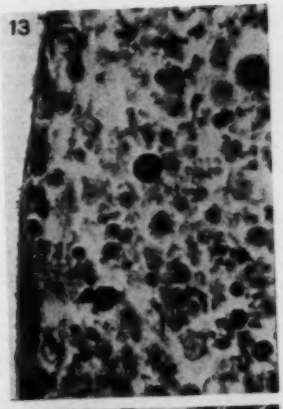

PLATE II

mass is less solidly packed with cells, and ectodermal cells have started to become arranged around the periphery (Pl. II, 14, 15).

Stage 5. Sixty hours after removal, the center of the mass of tissue is hollow. Well-defined ectodermal and endodermal layers have been formed around the periphery (Pl. II, 16, 17). The ectodermal cells are smaller than the ones in Stage 2 (Pl. I, 7).

Stage 6. Seventy-two to ninety-six hours after removal of the coenosarc fragments from the perisarc, regeneration occurs. This stage is characterized by the formation and emergence of new hydranths (Pl. I, 8–11). Hydranth formation is preceded by an aggregation of cells and later of pigment at the point of regeneration. Some of the fragments, although healthy in appearance, do not regenerate. They develop as far as Stage 5 (Pl. II, 16), at which time a heavy perisarc is secreted and development stops.

The changes which occur after removal of the coenosarc from the perisarc result in a general morphological dedifferentiation, namely, the formation of a spherical mass of coenosarc tissue in which the original ectoderm and endoderm are no longer clearly defined. Cellular dedifferentiation was not observed, no evidence being found that the cells return to an embryonic condition. The morphological dedifferentiation is followed by a redifferentiation, involving the reorganization of ectoderm and endoderm, formation of new perisarc, and subsequent regeneration. It is of interest to note that the time required for regeneration of the fragments is longer (72–96 hours) than the time required for intact stem segments (approximately 36 hours) at the same temperature. The additional time required for regeneration of the fragments is understandable when the time required for the initial dedifferentiation and early reorganization is taken into account.

The Rate of Oxygen Consumption of Coenosarc Removed from Different Levels of the Stem

In these experiments young unbranched stems were selected. The segments of stem used were 10 mm. long. The distal segments were taken from the region extending from 5 to 15 mm. proximal to the hydranth, and the proximal segments 20 to 30 mm. proximal to the hydranth. The coenosarc fragments were removed from these stem segments and kept in running filtered sea water until ready to be placed in the Warburg manometers. The fragments were placed in the manometers from 17 to 24 hours after removal from the perisarc. At this time the fragments have reached their greatest morphological dedifferentiation (Stage 3, Pl. II, 12). They have become spherical and are

protected from the mechanical shaking of the Warburg manometers by a thin, newly secreted perisarc. The rate of oxygen consumption was calculated on the basis of mm.^{3} O_2 used per hour per ten mg. of dry weight. The results are summarized in Table I. In most of the ex-

TABLE I

Oxygen consumption of distal and proximal coenosarc fragments removed from the perisarc. Rate = mm.^{3} O_2/hr./10 mg. dry weight. The temperature of the sea water during the experiments was 18.5±.02°C.

Description of coenosarc fragments			Oxygen consumption			
Exp.	No.	Region	O_2	Time	Dry weight	Rate
			mm.^{3}	hours	mg.	
RE 1	19	distal	48.5	10.75	0.494	91.0
	19	proximal	37.2	10.75	0.391	88.5
RE 2	15	distal	19.8	9	0.46	48.0
	15	proximal	23.4	9	0.69	37.6
RE 3	4	distal	6.6	22	0.224	13.4
	14	proximal	41.2	22	1.294	14.4
RE 4	18	distal	10.5	9	0.720	16.2
	17	proximal	10.8	9	0.735	16.3
RE 5	14	distal	13.1	8	0.378	43.5
	15	proximal	15.2	8	0.482	39.5
RE 6	19	distal	24.3	7	0.729	47.7
	19	proximal	19.6	7	0.694	40.5
RE 7	15	distal	18.2	11.5	0.764	20.7
	15	proximal	17.0	11.5	0.721	20.4

periments the distal rates are approximately the same as the proximal rates. The averages of the distal and proximal rates for the seven experiments are 40.1 and 36.7 respectively. The distal and proximal regions of stem segments with perisarc, however, show a distal-proximal gradient in rate of oxygen consumption (Barth, 1940*a*). This difference in rate is present after the stems are cut, and persists from 24 through 36 hours after cutting. The distal and proximal coenosarc fragments, however, show only slight difference in rate 24 hours after removal from the perisarc. This means that the coenosarc fragments must have lost the differential during the first 24 hours. Thus, the reorganizational changes in the coenosarc fragments involve a dedifferentiation of the physiological gradient present in the intact stem.

Apparently then, exposure of the coenosarc to sea water has an effect on the rate of regeneration. In order to clarify the nature of this effect, the rates of distal coenosarc fragments and distal stem segments may be compared. The average rate of oxygen consumption of distal coenosarc fragments is 40.1 as compared with 21.8 for distal stem segments, the latter average being calculated from data presented by Barth (1940*a*). If the dry weight of the stem perisarc represents even as high as 50 per cent of the dry weight of the stem, the rate of distal coenosarc fragments would still be as high as the distal rate of the stems. Thus, the distal coenosarc fragments consume oxygen at a rate at least as high as the distal stems. Since, in addition, distal and proximal coenosarc fragments respire at about the same rate, the effect of exposure of the coenosarc to sea water and the resultant dedifferentiation is to increase the rate of oxygen consumption of coenosarc from proximal levels of the stem up to the higher rate of the distal levels.

Polarity of the Regenerated Coenosarc Fragments

The polarity relationships exhibited by the regenerated coenosarc fragments are worthy of examination for comparison with stems, where only unipolar and bipolar forms regenerate after cutting. Uniform exposure of the coenosarc to sea water removes the possible complication of an environmental differential created by the presence of the perisarc. The regenerated fragments may be classified using the general terminology employed by Child (1927) for *Corymorpha*, the groupings being based upon the axial pattern developed by the fragments. The regenerated coenosarc fragments may be classified as unipolar, bipolar, bipolar-unipolar, multipolar, and apolar. The unipolar forms have regenerated a single hydranth on the rounded mass of tissue (Pl. I, 8). Bipolar regenerated fragments have formed hydranths at two opposite poles of the coenosarc mass (Pl. I, 9). The bipolar-unipolar forms have regenerated two hydranths from the same region of the coenosarc (Pl. I, 10). These may be two independent hydranths or two partially fused hydranths. The category "multipolar" is used to designate fragments which have regenerated more than two hydranths. This group includes regenerated fragments ranging from tripolar forms, to forms in which the entire surface of the coenosarc has become covered with tentacles (Pl. I, 11). In each of these categories are included forms in which the regenerated hydranths are not complete. Thus, a hydranth may be lacking in the number of oral or basal tentacles or lacking in any of the structures necessary for a complete hydranth. For the purpose of this analysis, however, it was not deemed necessary to subdivide the various

categories with respect to these irregularities. Many of the hydranths which form do not emerge from the new perisarc. These may be readily classified in the above groups, so that no distinction is made between emerged and non-emerged hydranths. The apolar forms are those fragments which fail to regenerate. They develop as far as Stage 5 (Pl. II, 16), form a thick perisarc, and remain in that condition.

The classification of the regenerated coenosarc fragments is arranged in Table II. The unipolar and multipolar forms make up the greatest percentage of the regenerants (78.6 per cent). The bipolar and bipolar-unipolar forms comprise a much smaller percentage of the total (21.4

TABLE II

Classification of the regenerated coenosarc fragments obtained after the removal of the perisarc. The observations were made at 96 hours. Percentage regeneration equals the number of a particular kind of regenerated coenosarc fragment divided by the total number of fragments which formed hydranths. The temperature of the sea water was 19±2°C.

	Description of the regenerated coenosarc fragments				No regeneration	
	Unipolar	Bipolar	Bipolar-unipolar	Multi-polar	Apolar	Dead
Number	73	26	9	56	50	166
Percentage Regeneration	44.5	15.9	5.5	34.1		

per cent). Since exposure of the naked coenosarc to sea water is a sufficient stimulus for hydranth formation (Zwilling, 1939), the formation of hydranths should be enhanced when the entire coenosarc is naked, and therefore in more direct contact with sea water and oxygen dissolved in the sea water. That this may be true is demonstrated by the relatively high percentage of multipolar regenerants (34.1 per cent) obtained after removal of perisarc. These multipolar forms are never obtained when the perisarc is left intact. The appearance of a high percentage of unipolars (44.5 per cent) suggests that in these cases the mass of tissue may have been exposed to a uniform gradient of oxygen in the sea water, for many of them became attached to the bottom of the dish at an early stage and the hydranths always formed away from the attached end. This interpretation is supported by Child's experiments with *Corymorpha* (1928) in which hydranths were regenerated at the upper surface of undisturbed cell aggregates. Coenosarc fragments develop in the same way, irrespective of the level of the stem from which they are removed.

Data for the regeneration of coenosarc fragments removed from three different levels of the stem are summarized in Table III. The coenosarc fragments of all three levels of the stem give rise to regenerants having similar types of polarity relationships. In addition, it may be noted that 96 hours after removal of the coenosarc from the perisarc, approximately

TABLE III

Classification of the regenerated coenosarc fragments removed from different levels of the stem. The observations were made at 96 hours after removal of the fragments from the perisarc.

	Description of the regenerated coenosarc fragments					No regeneration	
	Unipolar	Bipolar	Bipolar-unipolar	Multi-polar	Total	Apolar	Dead
Distal	10	4	2	12	28	14	18
Middle	12	3	1	13	29	19	12
Proximal	15	6	2	12	35	19	6

the same number of regenerants appear at all three levels. Thus, the gradient in the rate of regeneration present in *Tubularia* stems (Barth, 1938*b*, 1940*a*) apparently disappears when the coenosarc fragments are removed from the perisarc.

DISCUSSION

One of the chief difficulties in any attempt to analyze the rôle of the environment on regeneration is the inability to work with homogeneous systems. That hydroids have a gradient of metabolic activity has been well established. This gradient is developed, apparently as the result of an environmental differential, early in the development of the organism. Thus, Child (1925) has shown that a metabolic gradient is probably established as a result of the nature of the orientation of the egg during its growth. Further, once a gradient has been established, it may maintain itself in a uniform environment, and the gradient has therefore become a function of localized differences within the tissues themselves. Barth (1938*b*) demonstrated a gradient in the rate of regeneration along the length of the stem of *Tubularia*, distal segments regenerating at a higher rate than proximal segments. There is likewise a gradient of oxygen consumption of the parts of the stem (Barth, 1940*a*). Barth (1938*b*) also suggested that the dominance of distal over proximal levels of *Tubularia* stems might be interpreted as a compe-

tition for substance "S," the success of which is dependent upon the activity of enzyme "E." Thus, an organism living in a uniform external environment may nevertheless maintain its own heterogeneity once this heterogeneity has become established.

In order to determine more accurately the rôle of environmental factors on the origin of new gradients in regeneration, it is of importance to obliterate first any existing gradients in the animal tissues themselves. Some attempts in this direction have been made. Child (1928) found that cells of *Corymorpha,* when dissociated mechanically, will aggregate and establish new polarity relationships. *Corymorpha* stems, when subjected to toxic agents, may lose their established polarity relationships and form new gradients of regeneration (Child, 1927), the new gradients being produced by a differential exposure to the environment.

The experiments with expressed *Tubularia* coenosarc indicate an obliteration of the original polarity after the coenosarc is removed from the perisarc. This is borne out by the reorganizational changes which the coenosarc undergoes after removal. There is, at first, a morphological dedifferentiation, in which the mass becomes spherical and the ectoderm and endoderm are no longer clearly defined. Cellular dedifferentiation, however, was not observed. The morphological dedifferentiation is followed by a redifferentiation. The ectoderm and endoderm are reorganized and a new perisarc is formed. Subsequently, regeneration occurs. If the polarity of an organism is dependent upon a gradient of activity of some enzyme, as suggested by Barth (1938*b*), then it is quite likely that the activity of this enzyme is radically changed during the process of reorganization. That the initial polarity is lost, is evidenced also by the appearance of regenerated hydranths at the free surface of fragments which have become attached to the bottom of the dish, the regenerated hydranths having no necessary relation to the original polarity. It is further substantiated by the appearance of a high percentage of multipolar forms in which appear numerous and apparently unrelated polarity relationships. The appearance of these multipolar forms must mean that the original polarity is no longer extant; the exposure of the coenosarc to the sea water being sufficient to stimulate regeneration at many points on the uniform mass of tissue.

Correlated with the disappearance of the original polarity is the disappearance of the difference in oxygen consumption of tissues removed from distal and proximal levels of the stem. The sharp gradient of oxygen consumption found along the length of the stem (Barth, 1940*a*) is not exhibited by the excised fragments at the time when morphological dedifferentiation has reached its peak (17–24 hours after removal of the tissue). At this time the rates of oxygen consumption of dedifferenti-

ated coenosarc fragments from both distal and proximal levels of the stem are at least as high as the rate for distal stem segments. The loss of the gradient of oxygen consumption is due, therefore, to a general increase in rate. This increase in rate is probably stimulated by a high availability of oxygen to the coenosarc fragments, made possible by their removal from the perisarc. Thus, the removal of the coenosarc from the perisarc results in a reorganization involving not only morphological dedifferentiation but also a dedifferentiation of the physiological gradient. The end product of this process is a more homogeneous mass of *Tubularia* cells. The localized differences in the ability to regenerate, found in stems covered with perisarc, also disappear as a result of the morphological and physiological dedifferentiation. Coenosarc fragments removed from distal and proximal levels of the stem regenerate at the same rate and develop similar types of polarity relationships.

Expressed coenosarc, therefore, if used at the time when dedifferentiation is greatest (approximately twenty-four hours after removal from the perisarc), offers good biological material for studies of the environmental factors stimulating regeneration and for an investigation of the origin of polarity in regeneration.

Summary

The morphogenesis of coenosarc expressed from the perisarc of *Tubularia* stems is described. A series of structural changes occurs in the coenosarc, there being first a dedifferentiation of histological structure, followed by a redifferentiation culminating in the regeneration of new hydranths.

The gradient of oxygen consumption present in the stem of *Tubularia* disappears when the coenosarc is removed from the perisarc. This physiological dedifferentiation represents an increase as well as an equalization of oxygen consumption by coenosarc fragments from distal and proximal levels of the stem.

Concomitant with the morphological and physiological dedifferentiation, differences in the ability of distal and proximal levels of the stem to regenerate disappear. Distal and proximal coenosarc fragments regenerate at the same rate and develop similar types of polarity relationships.

The different kinds of regenerants obtained are described and classified on the basis of their polarity relationships. Evidences were given that these polarity relationships are new, and have no relation to the original polarity in the intact stem.

The value of using expressed coenosarc to study the effect of the environment on regeneration and on the origin of polarity in regeneration is discussed.

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THE RÔLE OF FERTILIZIN IN THE FERTILIZATION OF EGGS OF THE SEA-URCHIN AND OTHER ANIMALS

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INTRODUCTION

The striking phenomenon of the specific agglutination of spermatozoa by a substance obtained from the eggs has been described in a number of species of marine animals (see Lillie, 1919; Just, 1930; Tyler, 1940*a*). Lillie considered this substance, which he called fertilizin, to play a central rôle in the fertilization process, and developed a theory of the mechanism of fertilization based on the ability of fertilizin to combine with the spermatozoön and with some substance within the egg. One of the principal arguments for his views was the evidence that eggs of the sea-urchin which had been deprived of fertilizin lost their capacity for fertilization. In his first experiments (1914) the fertilizin was removed by prolonged washing of the eggs (*Arbacia*), combined in some cases with shaking to remove the jelly layer which he had shown (1913) to be heavily charged with fertilizin. Loeb (1914, 1915) raised the objection that the decrease in fertilizability was due to the aging and death of the eggs during the washing period of one to three days. He showed, on the other hand, that fresh eggs of *Strongylocentrotus purpuratus*, that had been deprived of their jelly layer (which he considered identical with fertilizin) by means of acidified sea water, would still give 100 per cent fertilization. Lillie (1915) repeated the acid sea water experiments with *Arbacia* and found the capacity for fertilization (per cent fertilized) to be much reduced as a result of the treatment. He also noted that some fertilizin could be obtained from the acid-treated eggs although the jelly layer appeared to be completely gone. Later (1921, footnote p. 16), with *Strongylocentrotus*, he found that acid-treated jellyless eggs could still be fertilized although there could not be obtained from these eggs sufficient fertilizin to agglutinate the spermatozoa. He interpreted that result to mean that "an amount of fertilizin insufficient for sperm agglutination is yet adequate for fertilization."

The present experiments resolve these differences as apparently being due to differences in amount of sperm employed for insemination. The

results show that jellyless, fertilizinless eggs can be fertilized but that they must be inseminated with much higher concentrations of sperm than are necessary for untreated eggs. The present, as well as some of the earlier work of the author (1939, 1940), also lends support to Lillie's view that fertilizin is concerned in the fertilization process, and some suggestions are made here as to its rôle. It is further shown that the sperm agglutinating property of fertilizin can be destroyed without altering its ability to combine with the sperm. An interpretation is offered of the temporary nature of the agglutination reaction in the sea-urchin and its more permanent nature in forms like the keyhole limpet. Evidence is also presented that fertilizin is not merely confined to those species of animals whose egg water causes iso-agglutination of sperm, but is more generally distributed and may very likely be universal.

Identity of Fertilizin with the Gelatinous Coat of the Egg

It has been shown (Tyler, 1940*a*) in experiments with the sea-urchin *Strongylocentrotus purpuratus* and the keyhole limpet *Megathura crenulata* that the sperm agglutinin (fertilizin) is located in the jelly layer surrounding the egg. On the rather reasonable assumption that the material of the jelly is a single substance, this means that fertilizin is identical with the jelly. In any event, the evidence showed that fertilizin is a component of the jelly layer and is not secreted by the ripe eggs. Removal of the jelly layer was readily accomplished by means of sea water acidified to between pH 4.5 and 3.5 and also by means of a 1 per cent solution of chymotrypsin in sea water. No fertilizin could be obtained from such jellyless eggs even after prolonged standing. When ripe eggs are allowed to stand in sea water, the jelly slowly goes into solution and the concentration of fertilizin in the solution increases. But this, it was shown, does not increase the total amount of fertilizin that can be obtained from the eggs. In other words, extraction of freshly shed eggs with acid sea water gives just as much fertilizin as that in the acid extract of eggs that had stood for some time in sea water plus that in the supernatant sea water.

Hartmann, Schartau and Wallenfels (1940) support the view that fertilizin is identical with at least a part of the material of the jelly layer. They found in *Arbacia pustulosa* that fertilizin is given off in repeated changes of sea water as long as remains of the jelly layer are present on the eggs. They also removed the jelly layer by means of a sperm extract containing an antifertilizin (see Frank, 1939; Tyler, 1939*a*, 1940*b*; Tyler and O'Melveny, 1941) and obtained no fertilizin from the treated eggs. Further evidence for this view is given by their finding that *Ar-*

bacia sperm agglutinate on the surface of the jelly layer of *Astropecten* eggs but fail to do so if the eggs are first treated with the sperm extract which forms a precipitation membrane on the surface of the jelly.

Additional evidence along this line is contained in some experiments by Evans (1940). He found that Roentgen radiation caused an immediate disappearance of the jelly from around the *Arbacia* egg. Using Janus green as a test for the presence of jelly in egg water, he found that after an irradiation of 59,000 *r* or more it could not be demonstrated in the egg water. He also noted that the agglutinating power of the egg water is greatly reduced after irradiation, and this agrees with Richards and Woodward's (1915) earlier results.

Fertilization after Removal of Fertilizin

The primary question concerning the rôle of fertilizin is whether or not its complete removal in a non-injurious manner renders the eggs non-fertilizable. This question was examined in some experiments with eggs and sperm of the west coast sea-urchin *Strongylocentrotus purpuratus*. Since, as the evidence shows, fertilizin is identical with, or at least a component of the gelatinous coat of the egg, its removal involves the dissolution of this coat. In *Strongylocentrotus*, the jelly is rapidly dissolved by placing the eggs in sea water acidified to between pH 3.5 and 4.5 (Tyler and Fox, 1940). If the eggs are not allowed to remain too long in the acid sea water, there is no visible sign of injury.

Although the jelly is colorless and transparent, its absence is readily noted by the fact that the eggs can then be brought into contact with one another by their surfaces (Tyler, 1940*b*, Fig. 1, *d*). When eggs of *Strongylocentrotus* are deprived of their jelly coat and washed, no detectable (by agglutination of sperm) amount of fertilizin can be obtained either by allowing them to remain for prolonged periods in sea water or by macerating and extracting them with various solvents (Tyler and Fox, 1940).

Upon insemination the jellyless eggs are capable of fertilization to the extent of 100 per cent, as Loeb (1914, 1915) and Lillie (1921) had reported for eggs of *S. purpuratus*. One typical experiment may be cited. Two 20 cc. samples of a 0.1 per cent suspension of fresh *S. purpuratus* eggs in sea water were taken and one of them acidified to pH 4.0. After 5 minutes both dishes of eggs were given a set of four washings with a total of 100 cc. of sea water, allowing the eggs to settle and 1 cc. of suspension to remain in the dishes between washings. The acid-treated eggs were observed to be deprived of their jelly. The addition of 0.05 cc. of a 1 per cent fresh sperm suspension gave 100 per cent

membrane elevation and cleavage in both the acid-treated and control eggs. Similar results were also obtained when the jelly was removed with chymotrypsin.

It may be concluded from this evidence that fertilizin is not essential for fertilization. However, such a conclusion is only valid if the fertilizin has in fact been completely removed from the treated eggs. That this may not be the case is indicated by other evidence and considerations presented below. But, even if it be assumed for the present that fertilizin is not essential for fertilization, the question may still be raised as to whether or not it is an aid to fertilization.

Fertilizin as an Aid to Fertilization

It is well known that the number of spermatozoa required for fertilization is in general much greater than the number of eggs present in the suspension, and as the number of spermatozoa employed for insemination is decreased, the percentage fertilization decreases. The factors responsible for this fact, that many more than one spermatozoön per egg must in general be present in the suspension in order for fertilization to succeed, have been examined by several investigators (Glaser, 1915; Lillie, 1915; Cohn, 1918; Morgan, 1927, p. 27 et seq.), and will not be discussed in any detail here. The present question is whether or not more spermatozoa are required for fertilization when fertilizin is removed from the eggs. This question was investigated with eggs and sperm of *Strongylocentrotus purpuratus* and the results do, in fact, show a decrease in "fertilizability" (increase in amount of sperm required for fertilization) upon removal of the jelly.

Table I lists the results of nine experiments in which the jelly was removed by means of acidified sea water or chymotrypsin. In all cases the eggs were washed after treatment and the pH brought back to that of normal sea water. In the table, cleavage rather than membrane elevation is taken as an index of fertilization inasmuch as the treated eggs often fail to form or to elevate fertilization membranes but may nevertheless cleave (see Tyler and Scheer, 1937). The amounts of sperm added are for convenience all given on the basis of a 0.01 per cent sperm suspension although actually the larger amounts of sperm were taken from more concentrated suspensions. In the different experiments there are, as the table shows, considerable variations in the amount of sperm required to give the same percentage fertilization of the control eggs. This may be due to variations in the condition of the sperm and eggs, in aging of the sperm at various dilutions, in temperature, etc. For the point in question, however, it suffices to compare simply the jellyless with

the control eggs in each horizontal line. The results show that with the larger amounts of sperm the jellyless eggs give practically the same percentage fertilization as the controls. But with smaller amounts there are considerable differences. Thus, with small amounts of sperm that give between 75 and 100 per cent fertilization in the control eggs, only 0 to

TABLE I

Fertilization of jellyless and normal eggs of *S. purpuratus* inseminated with various amounts of sperm. The egg suspensions contain 200 to 400 eggs in 5 cc. of sea water.

Experiment	Treatment for removal of jelly coat	Amount of 0.01 per cent sperm suspension added	Percentage cleavage	
			Jellyless eggs	Control eggs
		cc.		
1	30 min. in pH 4.5 sea water	0.1	5	95
		0.5	35	98
		2.0	99	99
2	30 min. in pH 4.0 sea water	0.05	0	20
		0.5	15	90
		5.0	95	95
3	5 min. in pH 3.5 sea water	0.2	0	25
		1.0	55	95
		5.0	100	100
4	10 min. in pH 3.7 sea water	0.2	10	85
5	10 min. in pH 3.9 sea water	0.1	20	100
		1.0	100	100
6	30 min. in 1 per cent chymotrypsin in pH 8 sea water	0.05	0.2	90
		1.0	45	95
7	30 min. in 1 per cent chymotrypsin in pH 8 sea water	0.1	0.1	75
		2.5	100	100
8	10 min. in 1 per cent chymotrypsin in pH 6 sea water	0.25	15	100
		2.5	100	100
9	10 min. in 1 per cent chymotrypsin in pH 6 sea water	0.5	45	95
		5.0	95	95

20 per cent is obtained in the jellyless samples. To get the same percentage fertilization as in the controls, the amount of sperm required for the treated eggs is roughly five to ten times greater. While the variations in the results do not permit an exact figure to be given for this ratio, it is clear that the differences are all in the same direction in each experiment and are well outside the limits of error. It should

also be noted here that, since sufficient sperm gives as much fertilization in the treated eggs as in the controls, there is no particular injurious action of the treatment involved.

Fertilizin as a Barrier to Fertilization

It appears then that the presence of fertilizin on the eggs is an aid to fertilization in that smaller amounts of sperm are required than in its absence. It might be supposed, then, that restoration of the fertilizin would eliminate the difference and that addition of fertilizin to normal eggs would lower the amount of sperm required for fertilization. Unfortunately, no way is as yet known by which the fertilizin can be restored in its normal state; that is, in the form of a gelatinous coat around the egg. When the jelly is dissolved in acidified sea water it does not go back on to the eggs upon neutralization of the suspension but remains in solution. One might, however, enquire whether or not the presence of fertilizin in solution in the egg suspension increases the fertilizing power of the sperm. This was examined with both jellyless and normal eggs, and it was found that, instead of increasing the fertilizing power of the sperm, the presence of fertilizin in solution had the opposite effect. In one experiment the fertilizin was restored in its original amount (but in solution) and in roughly ten times that amount to suspensions of naked eggs. Various amounts of sperm were used for insemination. The lowest quantity of sperm that gave 100 per cent fertilization in the jellyless controls gave only 15 per cent in the sample with original fertilizin content and 0 per cent in that with the ten-fold concentration. In an experiment with normal eggs the smallest amount of sperm that gave 100 per cent fertilization, gave about 35 per cent when an amount of fertilizin roughly equivalent to the content of the eggs was present in solution and 0 per cent when ten times that amount was present.

The presence of fertilizin in solution evidently acts as a barrier rather than an aid to fertilization. This action, it appears, is due to increase in amount of agglutination of sperm that occurs with increase in amount of fertilizin present in the solution. It is not merely the temporary locking up of the sperm in the agglutinates that causes the decrease in fertilizing power, but, as the next section shows, it involves a permanent effect of the fertilizin on the sperm.

Loss of Fertilizing Power as a Result of Agglutination

F. R. Lillie (1913) showed that the agglutination of sea-urchin sperm by egg water (fertilizin) is temporary. On testing the sperm after reversal of agglutination, he found them to have about half the fertiliz-

ing power (fertilized half the percentage of eggs) of the control sperm suspension. He also noted (1919) that after reversal of agglutination the sperm cannot be re-agglutinated. I have confirmed these findings with *S. purpuratus* and have obtained a much greater reduction in fertilizing power of the sperm.

In twelve experiments that were run, the sperm was agglutinated with sufficiently strong egg water, so that further addition of egg water, after reversal, gave no visible agglutination. The agglutination usually lasted 30 to 40 minutes, Insemination with amounts of sperm that were well above the control minimum for 100 per cent fertilization gave in all tests with the agglutinated and reversed sperm between 0 and 3 per cent fertilization. To obtain the same percentage fertilization with the control as with the treated sperm was found to require between a forty- and a two-hundred-fold reduction in the amount of control sperm used for insemination. The possibility was also examined that the reversed sperm might be more capable of fertilizing jellyless eggs, but the results were negative.

Along with this reduction in fertilizing power of the sperm there is no visible sign of any injurious effect after reversal of agglutination, nor is there any reduction in the activity of the sperm. In fact, the egg water, as is well known, increases the activity of the sperm and as measurements of respiratory rate showed (Tyler, 1939*b*) the increase persists long after the reversal of agglutination. The experiments show, then, that sperm which have been agglutinated are, after spontaneous reversal, incapable of fertilization. The small percentages of fertilization that result when large amounts of treated sperm are used are evidently due to the fact that some spermatozoa in the treated suspensions may escape being agglutinated.

It may be concluded, then, that some change is produced in the spermatozoa, as a result of their reaction with fertilizin, which, although essentially non-injurious, renders them incapable of fertilizing normal eggs. This change might occur during the initial reaction or upon the spontaneous reversal of the agglutination.

The Spontaneous Reversal of Sperm-Agglutination in Sea-Urchins

The temporary nature of the agglutination reaction exhibited by sea-urchin sperm in egg water is an exceptional affair. In the usual serological reactions, the agglutination of various types of cells (blood cells, spermatozoa, bacteria, etc.) by their antisera does not spontaneously reverse, but persists indefinitely. Natural agglutinins, too, such as the

blood group agglutinins in humans, give permanent agglutination which can only be reversed by special treatment. It is of interest, then, not only in connection with fertilization, but in regard to the nature of agglutination reactions in general, to consider the possible causes of the spontaneous reversal.

We shall use as a basis of the present discussion the lattice or framework theory of Heidelberger (1938) and Marrack (1938). This theory postulates that in agglutination as well as precipitation reactions the antigen and antibody are structurally complementary and both are multivalent in regard to their combining groups. Thus one molecule of antigen may combine with more than one molecule of antibody which in turn may combine with more than one molecule of antigen and so build up large aggregates. Where both of the complementary substances are in solution, precipitation results. Where one is present as the surface of the cell, agglutination occurs. The following interpretations may then be suggested for reversal of agglutination in the sea-urchin. (1) The fertilizin molecules plus the combined antifertilizin split off from all of the spermatozoa, leaving neutralized fertilizin in the suspension. (2) They split off at some, rather than all, combining sites in such a way that each (completely neutralized) fertilizin molecule remains attached to not more than one spermatozoön. (3) The fertilizin molecules are split by the action of the sperm leaving univalent fragments combined with the antifertilizin on all the spermatozoa.

All three of these interpretations can account for the failure of reagglutination and the loss of fertilizing capacity on the part of the reversed sperm. Attempts were made to eliminate one or another of these possibilities but the experiments were inconclusive and need not be described here. However, some new findings and further consideration of earlier work lend support to the third interpretation.

It was shown (Tyler and Fox, 1940) that fertilizin of the keyhole limpet is much more resistant than that of the sea-urchin to inactivation by heat and proteolytic enzymes and that this greater stability correlates with the more permanent nature of the agglutination reaction in that form. That the difference is not due to differences in the relative amounts of fertilizin involved is evident by the fact that the reaction is of long duration in the keyhole limpet even when weak fertilizin solutions are employed, whereas it does not in the sea-urchin surpass a maximum of very much shorter duration when the strongest available fertilizin solutions are added. This suggests then that, in the sea-urchin, the combined fertilizin may be broken down fairly rapidly by action of the sperm. It has also been noted that when fertilizin solutions are heated or treated with proteolytic enzymes there is at first a small but definite

increase in activity followed by the gradual inactivation. This suggested the possibility that the fertilizin is first split into smaller but still multivalent molecules. Such behavior is not unique for it has been frequently noted with immune antibodies (see Marrack, 1938; Zinsser, Enders and Fothergill, 1939; Petermann and Pappenheimer, 1941) and the altered agglutinin is termed "agglutinoid." It seemed possible then that, by careful inactivation of fertilizin solutions, univalent fragments might be obtained. The "univalent" fertilizin should be incapable of causing agglutination, but should inhibit subsequent agglutination by untreated fertilizin. It should also be expected to be effective in destroying the fertilizing power of the sperm. As will be shown in the next section, both of these effects have been obtained with heat-treated fertilizin solutions. This, then, lends support to the third interpretation of the spontaneous reversal of agglutination; namely, that the fertilizin molecules are split and the univalent fragments remain attached to the combining groups on the sperm.

"Univalent" Fertilizin

In five experiments concentrated solutions of *S. purpuratus* fertilizin that had been purified by previously described methods (Tyler and Fox, 1940) were heated at 90° to 100° C. just up to the time at which the agglutinating activity had practically disappeared. Sperm was then added to samples (at room temperature) of (*A*) the heated solutions, (*B*) the control solutions, and (*C*) sea water, the relative amounts being such that complete agglutination (no reaction to additional fertilizin after reversal) occurred in the control solution. When unheated fertilizin was added to samples of the sperm in *A*, there was either a very weak reaction or no visible agglutination at all. After reversal of agglutination in *B*, normal eggs were inseminated with various amounts of the sperm suspensions. When amounts of sperm were used that, in the case of the sea water controls, *C*, were near the minimum for 100 per cent fertilization, the *A*-sperm gave 0 to 5 per cent (av. 0.5 per cent) and the *B*-sperm gave 0 to 1 per cent (av. 0.2 per cent) fertilization. A further control was run in those experiments where *A*-sperm showed a weak agglutination reaction upon addition of unheated fertilizin. This was done by diluting the control fertilizin to a concentration giving a similar reaction and adding sperm to the dilute solution at the same time and in the same relative amounts as employed in the other solutions. The fertilizing capacity of the sperm in the diluted fertilizin was found to be only slightly lower than that of the sea water control sperm. An absorption experiment was also performed by the addition of excess

sperm to a sample of the heated fertilizin solution and, after centrifugation, the active agent was found to have disappeared from the supernatant solution.

The results show, then, that the agglutinating property of fertilizin can be destroyed without altering appreciably its capacity to combine with the sperm. The heated fertilizin is usually somewhat weaker than the control in its ability to prevent subsequent agglutination and in its ability to destroy the fertilizing power of the sperm. This most likely means that a small amount of the fertilizin is more completely degraded during the heat treatment. It is clear, however, that by careful heat treatment a modified (non-agglutinating) fertilizin can be produced that differs only slightly, if at all, in its ability to combine with the sperm. Since according to the modern theory a specific agglutinating substance is assumed to be multivalent in respect to its specific combining groups, it is reasonable to consider the non-agglutinating substance in this case univalent.

The formation of univalent fertilizin may be assumed to involve the splitting of the molecule into fragments each of which contains a single combining group or it might involve the splitting off of the combining groups alone. In the latter instance the active agent would be expected to be of small molecular size. Dialysis tests showed, however, that the active agent is incapable of passing through a cellophane membrane. The first assumption appears then to be the more likely one. Other properties of the active agent have not as yet been studied except for a preliminary test of its inactivation by heat. It is inactivated in about one and one-half to three times the time required for destruction of the agglutinating property of the original fertilizin.

Fertilizin in Animals not Exhibiting Iso-Agglutination of Sperm

Lillie (1919) and Just (1930) assumed that eggs of all species of animals possessed fertilizin, although they, themselves, had shown that in many species there is no detectable agglutination of sperm by homologous egg water. They regarded the agglutination reaction simply as an indicator for the presence of fertilizin, but they did not offer any evidence or tests that would demonstrate an analogous substance in the absence of the clumping reaction. The present concept of univalent fertilizin has led to the demonstration of specific sperm-combining substances in species in which the agglutination reaction is lacking. If, in a particular species of animal, the fertilizin obtained in the egg water is univalent, then it should give no agglutination of homologous sperm, but it should destroy their fertilizing capacity.

This point was examined in the starfish *Patiria miniata* and in the gephyrean worm *Urechis caupo*. In the starfish, concentrated egg water causes no agglutination of homologous sperm. In *Urechis* there may occasionally be a weak reaction. Concentrated egg waters were prepared from eggs of these two species by extraction with pH 4 sea water. Sperm was then added to the neturalized homologous and heterologous egg waters as well as to sea water and after a few minutes various amounts were taken for insemination of the homologous eggs. In all cases there was found to be a great reduction in the fertilizing capacity of the sperm treated with homologous egg water, while that treated with heterologous egg water showed approximately the same fertilizing capacity as the sea water controls. A typical experiment may be cited. Concentrated *Patiria* and *Urechis* egg waters were prepared from 10 per cent egg suspensions. One part of a 1 per cent *Patiria* sperm suspension was added to nine parts of (*A*) *Patiria* egg water, (*B*) *Urechis* egg water and (*C*) sea water. The same was done with a one per cent suspension of *Urechis* sperm. Insemination of homologous eggs (approximately 200 eggs in 5 cc. of sea water) with 0.05 cc. of these mixtures gave for *Patiria* no fertilization with *A*, 100 per cent with *B* and 99 per cent with *C*. For *Urechis* the results were 100 per cent with *A* and *C* and 0 per cent with *B*.

These results then lend support to the view of Lillie and Just that fertilizin is of general distribution in animals. When appropriate material is available, the investigations will be extended. For the present it is evident in two species of animals that a specific sperm-combining substance is obtainable from the eggs and, since the substance has no agglutinating action on homologous sperm, it may be termed univalent fertilizin.

Discussion

It has been shown that fertilizin, when present in the form of a gelatinous coat, is an aid to fertilization in the sea-urchin. It would also appear from the experiments that fertilizin is not entirely essential to fertilization. But this assumes that all of the fertilizin is removed upon removal of the jelly. While no detectable fertilizin is obtainable from the jellyless eggs, it is quite conceivable that it may be present in combined form on the surface of the egg. It has been shown (Tyler, 1940*b*) that there is an antifertilizin below the surface of the egg and it would be reasonable to assume that the surface of the egg is composed of a fertilizin-antifertilizin complex. Upon removal of the jelly, this combined fertilizin would remain as a monomolecular layer with free spe-

cific combining groups on its outer surface. In support of this view may be cited the observation of Frank (1939) that jellyless as well as normal sea-urchin eggs can be agglutinated by means of an antifertilizin obtained from the sperm. The possibility may then be admitted that fertilizin is indispensable for fertilization but further evidence along this line would be desirable before any attempt is made to develop a theory of fertilization with it as an essential agent.

In regard to the manner in which fertilizin may act as an aid to fertilization there are several possibilities. In the first place it is clearly not merely the greater volume due to the presence of the jelly that is involved, since the spermatozoön must, in any event, reach the surface of the egg for fertilization to ensue. It is possible that the gradient produced, as the jelly slowly goes into solution, exerts a chemotactic effect on the sperm. There is, however, still no general agreement as to chemotaxis. Hartmann (1940) reports demonstrating such action of fertilizin by means of the Pfeffer capillary method, whereas Cornman (1941) could obtain no positive results with that method.

Another possibility is that the jelly serves as a trap for the sperm. This appears reasonable on the basis of the fact that the spermatozoön reacts with fertilizin in solution. One may suppose that, while most of the fertilizin is in the form of a jelly, some of it is in solution in the interstices; or that even as a gel there are some free combining groups available. The formation of a precipitation membrane on the surface of the jelly by the action of antifertilizin (Tyler, 1940*b*) is more readily explainable on the basis of the latter assumption. Trap action would help to explain how fertilizin (as a jelly) acts as an aid to fertilization, since it would restrict the random movements of the spermatozoa to a small volume and thereby increase the chance of fertilization. However, other and more quantitative experiments are needed before decision can be made as to whether or not it alone can account for greater fertilizability of the normal in comparison with the jellyless eggs.

Another possibility is that some structural property of the jelly causes the sperm to approach so that its long axis is normal to the surface. While observations (*see* Morgan, 1927; Chambers, 1933) indicate that a radial approach is more favorable for fertilization, it has not definitely been shown that oblique approach and contact with the surface results in failure of sperm entry.

The possibility should also be considered that the greater fertilizability of the normal eggs is due to the activating effect of fertilizin on the sperm. But before decision can be made as to the value of this factor, it would be important to know that there is no corresponding decrease in the fertilizable life of the sperm.

In connection with these possibilities, it must be recalled that after the sperm has reacted with fertilizin in solution it is incapable of fertilization and that, probably because of this, the presence of fertilizin in solution in a suspension of eggs acts as a barrier to fertilization. Thus excess sperm is required to take up the fertilizin in solution and leave uncombined sperm available for fertilization. It is evident that in normal fertilization the spermatozoön must reach the surface of the egg before the inhibiting action of the fertilizin surrounding the egg has taken place. If, as suggested above, fertilizin in the form of a jelly has only a few superficial combining groups available, it is quite conceivable that they may serve as the initial trap for the sperm but would not be sufficient to neutralize all of the reacting groups on the sperm before the latter has reached the surface of the egg. The increased activity of the sperm upon reaction with fertilizin would also aid its reaching the surface before the fertilization-inhibiting reaction went to completion. While this seems to be the most likely interpretation, it requires considerably more experimental support. Also, it appears that the information so far available does not warrant a detailed discussion of Lillie's theory of fertilization, nor of the recent views of Hartmann (1940), nor of the development of a new theory of the exact function of fertilizin and other specific substances.

It has been shown in the present work that appropriate treatment of sea-urchin fertilizin converts it into a non-agglutinating agent that is still capable of reacting specifically with the sperm. On the basis of the lattice theory of agglutination reactions this altered fertilizin may, quite legitimately, be designated a univalent substance. It was also shown that the egg waters of certain species of animals that do not contain specific sperm agglutinins nevertheless contain specific sperm-combining substances which may likewise be designated univalent. The absence of the agglutination reaction in many species of animals does not, then, mean the lack of fertilizin, if by that term we mean simply a substance that reacts specifically with the sperm.

This concept may also be extended to problems in general immunology. It is well known that certain animals, such as the rabbit and the horse, readily produce upon immunization, specific agglutinins and precipitins. Others, such as the mouse and the rat, produce little or none but do form protective or neutralizing antibodies. It may be suggested, then, that the antibodies produced in the latter species are principally or entirely of the univalent type. This possibility can be readily tested experimentally;—cells treated with the univalent antibodies should be rendered incapable of being agglutinated by the specific agglutinating antibodies obtained in the former species.

SUMMARY

1. It has been shown in the sea-urchin that the presence of fertilizin, in the form of the jelly coat of the egg, serves as an aid to fertilization. In solution it acts as a barrier to fertilization.

2. Confirmation is presented of Lillie's finding that sea-urchin sperm cannot be re-agglutinated after reversal of an initial agglutination. It is also shown that the reversed sperm are incapable of fertilization.

3. Appropriate heat treatment converts fertilizin into a substance that does not cause sperm agglutination but still combines with the sperm as shown by the inability of the sperm to be subsequently agglutinated by ordinary fertilizin and by loss of fertilizing power. In accordance with the assumption of multivalency in the lattice theory of agglutination, the modified fertilizin is assumed to be univalent. It is found to be non-dialyzable.

4. In the starfish and in *Urechis* the egg water is shown to contain a specific sperm-combining substance (univalent fertilizin) that is incapable of causing iso-agglutination of sperm.

5. Of various interpretations of the spontaneous reversal of agglutination in the sea-urchin, a splitting of the fertilizin into univalent fragments is considered the most likely.

6. Reasons are presented for holding open the possibility that fertilizin plays an indispensible part in fertilization. Various possible explanations as to the manner in which it serves as an aid to fertilization are discussed and that involving trap action is considered the most likely.

7. It is suggested that some species of animals produce upon immunization only, or principally, univalent antibodies and a method of determining this point is offered.

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SPECIFICITY AND HOST-RELATIONS IN THE TREMATODE GENUS ZOÖGONUS [1]

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The genus *Zoögonus* was erected by Looss (1901) to contain *Z. mirus,* (as type), and *Distomum viviparum* Olsson, 1868. *Zoögonus mirus* was described from two specimens found in the intestine of *Labrus merula* at Trieste. The worms measured 1.55 mm. in length and about 0.45 mm. in width. The following year, Odhner (1902) designated *Zoögonus viviparus* (Olsson, 1868) Looss, 1901 as type of a new genus *Zoögonoides.* In this paper he redescribed *Distomum rubellum* Olsson, 1868, and transferred the species to *Zoögonus.* Like Olsson, he found the parasites in *Labrus berggylta* (syn. *L. maculatus*) from the west coast of Sweden, but the examination of twenty fishes at the Zoölogical Station of Kristineberg disclosed only two infections and only a few worms were obtained. The specimens were yellowish in color, 1.1–1.4 mm. in length and 0.45 mm. in greatest width. *Zoögonus rubellus* was distinguished from *Z. mirus* on the size of suckers and length of the miracidia. Although Looss and Odhner were two of the ablest students of the trematodes, their observations on *Zoögonus* were limited to the study of very few specimens.

The specific identity of *Z. mirus,* questioned by competent investigators, still remains an unsolved problem. Goldschmidt (1902, 1905), after comparing specimens collected at Trieste with the descriptions of *Z. mirus* and *Z. rubellus,* stated that there were no morphological differences. The dimensions of suckers and miracidia, characters used by Odhner to separate the species, were found to be variable and hence invalid as specific criteria. Furthermore, Goldschmidt was unwilling to differentiate the species on the presence or absence of yellow pigment in the tissues. Nicoll (1909), who reported *Z. rubellus* as consistently abundant in *Anarhichas lupus* from St. Andrews Bay, described the worms as pale yellow in color, 0.75–1 mm. in length and about one-half

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as wide as long. Referring to the descriptions of Looss, Odhner and Goldschmidt, Nicoll stated, "My specimens agree best with Goldschmidt's description." It is significant that the worms studied by Nicoll and Odhner came from the same region, whereas Goldschmidt's material was collected in the Mediterranean and presumably was identical with that of Looss. Concerning specific determination, Nicoll expressed the opinion that, "Looss's *Zoögonus mirus* is in all probability identical with Odhner's *Z. rubellus* . . . at any rate, features sufficient to distinguish them are not at present apparent."

In a systematic review of the family Zoögonidae, Odhner (1911) maintained that the specimens of *Zoögonus* from the Mediterranean and North Sea are specifically distinct. After collecting material from both locations, he distinguished the two species on differences in size and color, size of suckers and shape of sucker cavities, location of the acetabulum, length of the digestive ceca, position of the cirrus sac, and number of eggs in the uterus. Odhner stated that extended specimens of *Z. mirus* never exceed 0.6 mm. in length and 0.2 mm. in width, whereas similar specimens of *Z. rubellus* measure 0.9–1.2 mm. in length and about 0.25 mm. in width. He noted that the pharynx and the miracidia are approximately the same size in the two species, but appear to be larger in the smaller specimens of *Z. mirus*.

The specimens of *Z. mirus* studied by Odhner were apparently contracted and probably not entirely mature. His statement that extended worms never exceed 0.6 mm. in length cannot be accepted, since the type specimens described by Looss measured 1.55 mm. in length. The number of eggs in the uterus obviously is correlated with the degree of sexual development, and the other features employed by Odhner to differentiate the species manifest so much variation that subsequent authors have disagreed on the identity or distinctness of the European species of *Zoögonus*. It is apparent that morphology of the adult stages is inadequate for a final solution of the problem.

Although a beginning has been made on the life history of *Zoögonus*, information from this source is still too fragmentary to permit final specific determination. In a series of papers, Timon-David (1933, 1934, 1936, 1938) described encysted metacercariae, identified as larvae of *Z. mirus*, in sea-urchins collected in the Gulf of Marseilles and along the coast of Roussillon. The metacercariae were found only in the muscles of the lantern of Aristotle. The degree of infection was variable and from one to sixty cysts were recovered from individual urchins. The incidence of infection in *Paracentrotus lividus* reached 50 to 60 per cent, a somewhat lighter infection was common in *Sphaerechinus granularis*, only a few cysts were recovered from *Arbacia aequituberculata*,

whereas no infection was observed in *Echinus acutus* or *Psammechinus microtuberculatus*. All the parasites apparently belonged to a single species. The cysts increased in size with the development of the metacercariae, and measured from 0.15 to 0.25 mm. in diameter. A cyst (1934, Fig. 1), fixed in picro-formol-alcohol solution under moderate compression, measured about 0.4 mm. in diameter, according to the scale accompanying the drawing. The cyst wall measured 0.003 mm. in thickness and was not surrounded by a connective tissue capsule. The worm was bent upon itself, with the dorsal surface applied to the wall of the cyst. Released from their cysts, the mature larvae averaged 0.6 mm. in length and 0.2 mm. in width. Specimens from *P. lividus*, *A. aequituberculata* and *S. granularis* (1934, Figs. 2, 3, 4), fixed in extended condition, measured 0.83, 0.93 and 0.66 mm. respectively (length calculated from scales accompanying the figures). Timon-David (1936) reported that metacercariae fed to *Blennius gattoruginae* excysted and persisted in the intestine for 45 days. Such a specimen, figured in the report, measured 0.64 mm. in length and was little if any farther advanced in development than larvae freshly removed from their cysts. The observation of Timon-David, that remains of sea-urchins were frequently present in the intestine of *Labrus merula*, supports his opinion that the metacercariae from the urchins are actually larval stages of *Z. mirus*. In his (1934) paper he recalled that the development of the miracidia has been known since the accounts by Looss (1901), Goldschmidt (1902) and Wassermann (1913), but that the first intermediate host remains as yet unknown. He expressed the belief that the cercarial stages are to be sought among the gastropods of the region.

In a report on larval trematodes from the region about Roscoff, Finistère, Stunkard (1932) described a tailless cercaria, *C. reticulatum* from *Nassa reticulata*, which shows such remarkable morphological agreement with the metacercariae described by Timon-David that the two must be closely related and may possibly belong to the same species. One item in the description of Stunkard requires correction. In the figure, the pharynx is represented as only a short distance in front of the acetabulum, whereas notes made at the time state that the pharynx is situated about midway between the suckers.

A single species of *Zoögonus* has been recorded from the Atlantic coast of North America. It was first described in the cercarial stage by Leidy (1891), who named it *Distomum lasium*. The larvae develop in *Nassa obsoleta*. Subsequent studies on the cercaria were reviewed by Stunkard (1938), who completed the life cycle. The cercariae encyst in polychaete annelids, principally *Nereis virens*. Natural infections were found in eels and sexually mature specimens were recovered after ex-

perimental infection of the eel and toadfish. Comparison of adult specimens with descriptions of *Z. rubellus* and *Z. mirus* provided no positive basis for specific distinction and so notwithstanding certain differences in hosts, life cycle, and morphological details, Stunkard regarded the American specimens as specifically identical with *Z. rubellus* and *Z. mirus*.

Subsequent studies of the European species carried on at the Station Zoologique de Wimereux in 1939 and of the American species at the Marine Biological Laboratory in Woods Hole during 1940, have yielded such discordant results that the question of specific identity must be reconsidered. The results of these observations were reported in abstract (Stunkard, 1940).

A sojourn at Wimereux, France, from July 8 to August 14, 1939, provided an opportunity to reexamine European phases of the *Zoögonus* problem. Metacercariae were found in *Psammechinus miliaris*, the common sea-urchin of the region. Urchins were collected at different locations from the Port de Boulogne to Ambleteuse, a stretch of some ten kilometers. The heaviest infection appeared in specimens from the Port de Boulogne where encysted larvae were recovered from more than 50 per cent of the urchins dissected. Different individuals harbored from one to thirty-six metacercariae. Lighter incidence and degree of infection were found in urchins taken between Boulogne and Ambleteuse. All of the larvae appeared to belong to a single species.

The metacercariae were encysted in the muscles and connective tissue of the lantern of Aristotle. The cysts were transparent, with no obvious reaction of the host to the parasite. The cyst wall was thin, colorless and very tough. The cysts measured from 0.2 to 0.28 mm. in diameter. The larvae, freed from their cysts, measured 0.42 to 0.65 mm. in length and 0.15 to 0.22 mm. in width. The cuticula was spined, although the spines were reduced in size and number behind the level of the testes. Larvae fixed under cover-glass pressure measure up to 0.95 mm. in length and a small one (Fig. 3), apparently equally mature but fixed without pressure, is only 0.37 mm. in length. When the worms are fixed without compression, the preacetabular portion bends ventrad, so that in mounted specimens the oral sucker may appear above or below and immediately in front of the acetabulum (Figs. 1, 2, 3). A representative specimen, fixed under slight pressure and shown in Fig. 4 is 0.75 mm. in length. The acetabulum, situated near the middle of the body, measures 0.08 by 0.086 mm. in diameter. The pharynx is located about midway between the suckers and measures approximately 0.06 mm. in diameter. When the specimen was extended the pharynx tended to be longer than wide. Its anterior end is dentate and the nuclei of the organ are concentrated

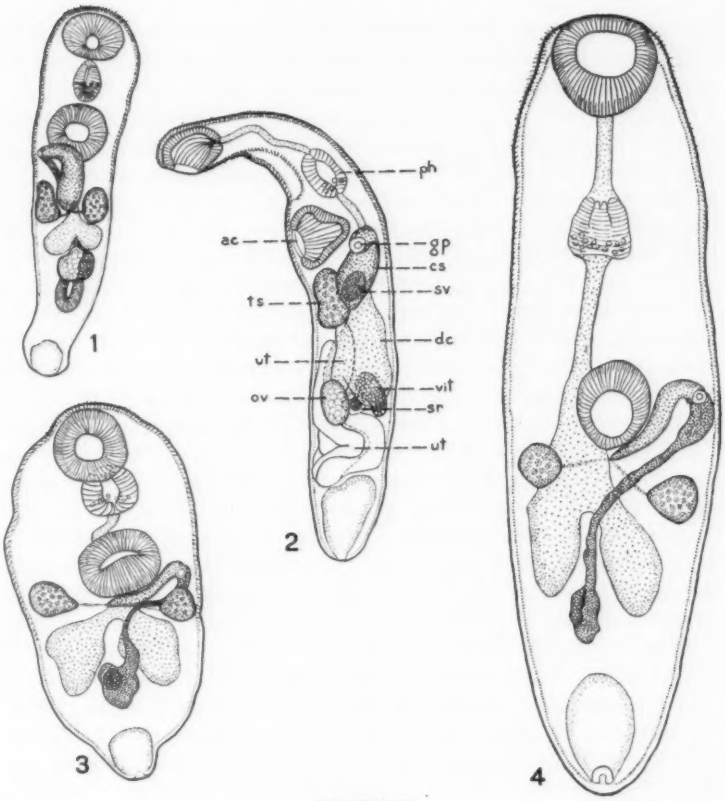

PLATE I

Abbreviations

ac	acetabulum	*sr*	seminal receptacle
cs	cirrus sac	*sv*	seminal vesicle
dc	digestive cecum	*ts*	testis
gp	genital pore	*ut*	uterus
ov	ovary	*vit*	vitellaria
ph	pharynx		

All figures are drawn to the same scale from fixed, stained and mounted specimens.

FIG. 1. Mature metacercaria, developed six weeks in *Nereis virens,* fed to a toadfish and removed two days later; Woods Hole. One of the largest specimens, fixed without compression, anterior end bent ventrad; dorsal view.

FIG. 2. Sexually immature specimen, developed six weeks in *N. virens* and six weeks in an eel; Woods Hole. Shows preacetabular ventral bending of body in specimens fixed without compression; lateral view from left side.

FIG. 3. Mature metacercaria from *Psammechinus miliaris;* Wimereux. One of the smallest specimens, fixed without compression, anterior end bent ventrad; ventral view.

FIG. 4. Mature metacercaria from *P. miliaris;* Wimereux. An average size specimen, fixed under cover-glass pressure, 0.75 mm. long; ventral view.

in its posterior half. The excretory system was worked out completely and agrees in detail with that of *Cercariaeum reticulatum* and with that of the American species of *Zoögonus*.

Measurements of the metacercariae from Wimereux do not differ greatly from those given by Timon-David for metacercariae from sea-urchins in the Mediterranean. The suckers in my specimens are slightly smaller than those measured by Timon-David, although his figures show the apertures of the suckers to be wide open and the specimens may have been more flattened. He figured the acetabulum slightly behind the middle of the body, whereas in my material it tends to lie slightly in front of the middle, although this feature is variable and changes with the extension or retraction of anterior and posterior regions of the body. Consequently, it appears likely that the specimens from Wimereux are specifically identical with those from the Mediterranean.

After discovery of the metacercaria, attempts were made to find the other stages in the life cycle of the European species of *Zoögonus*. Examination of many fishes, including several specimens of *Labrus sp.*, were fruitless. In view of the possibility, expressed previously, that the tailless larva, *C. reticulatum*, may represent a stage in the life history, wide search was made for it. The host, *Nassa reticulata*, is abundant in the region but the examination of more than 1200 specimens did not yield a single infection with tailless cercariae. Over 800 of these snails were collected from mud between rocks of the breakwater in the Port de Boulogne, and sea-urchins taken from these rocks were heavily infected with metacercariae. Other mollusks examined for *Zoögonus* larvae, with negative results, included 240 *Mytilus edulis*, 146 *Barnea candida*, 28 *Tapes pullastra*, 10 *Ensis sp.*, 240 *Patella vulgaris*, 35 *Purpura lapillus*, 34 *Littorina obtusa*, 86 *L. rudis*, 56 *L. littorea*, and 45 *Gibbula cineraria*.

To determine whether annelids as well as echinoderms harbor metacercariae of the European species of *Zoögonus*, worms were carefully dissected under a binocular microscope. The examination of 14 *Eunereis longissima*, 12 *Nereis errorata* and representatives of other unidentified polychaetes from the Port de Boulogne did not disclose any metacercariae.

In view of the failure to discover other stages in the life cycle of *Zoögonus* in the Wimereux area, the origin of the infection in the sea-urchins and subsequent fate of the larvae are entirely problematical. The completion of the life history by trematode parasites would be difficult in this region since the tides have an amplitude of eight to ten meters and the collecting grounds, exposed at low tide, are covered by an enormous volume of water six hours later.

Comparison of the larval stages of *Zoögonus* found along the north coast of Europe and the eastern coast of North America shows slight but apparently significant differences. Cercariae from *Nassa reticulata* at Roscoff average slightly larger than those from *N. obsoleta* at Woods Hole and the suckers are also larger. In the European form the ranges of size are: acetabulum, 0.068–0.076; oral sucker, 0.076–0.085; and pharynx, 0.03–0.04 mm., whereas measurements of the corresponding structures in the American form are: 0.062–0.075; 0.043–0.055; and 0.022–0.028 mm. Moreover, in the European form the prepharynx is relatively shorter and the pharynx is about midway between the suckers, whereas in the American form the pharynx is farther posteriad and frequently overlaps the acetabulum. The metacercariae from Wimereux and from Woods Hole show the same differences as the cercariae from the two regions. Cysts of the European form are slightly larger, the metacercariae are larger (compare Figs. 1, 2, and 3) and the relative sizes of suckers persists. Average measurements of ten specimens from the two localities give the following sizes (dimensions of Wimereux specimens first, of Woods Hole specimens second): acetabulum 0.085 *vs.* 0.075; oral sucker 0.09 *vs.* 0.065; pharynx, 0.06 *vs.* 0.042 mm.

To determine whether the American species of *Zoögonus* may occur in sea-urchins as well as polychaete annelids, attempts were made at Woods Hole in the summer of 1940 to infect urchins with *Distomum lasium* (= *C. lintoni*). Many freshly dredged urchins, both *Arbacia punctulata* and *Strongylocentrotus drobachiensis*, were dissected with negative results. Since enormous numbers of these animals have been used during the past forty years for embryological and other studies without the reported finding of metacercariae, natural infection with trematode larvae must be absent or very slight. Portions of dissected urchins, including the denticles and attached tissues, were placed in dishes of sea water with scores of naturally emerged cercariae of *Zoögonus*. The larvae crawled about over the tissues but did not penetrate or encyst. They were not attracted toward intact sea-urchins or dissected portions of them. Single urchins were exposed for several hours during the day in finger bowls to hundreds of cercariae and maintained during the intervening time in large aquaria. Dissection of the urchins later did not disclose any metacercariae.

Although the factors concerned with infection of the secondary intermediate host are virtually unknown, it is apparent that experiments devised to secure experimental infection in the laboratory must approximate natural conditions as closely as possible. Accordingly, on August 16, 1940, twenty specimens of *N. obsoleta* from which cercariae were emerging in large numbers were placed in each of two aquaria. Fifteen speci-

mens of *A. punctulata* were added to one aquarium; five specimens of *A. punctulata* and five specimens of *S. drobachiensis* to the other. After an interval of a week, dissection of the urchins was begun. No infection was found in *S. drobachiensis* but *Zoögonus* larvae were recovered from eleven specimens of *A. punctulata*. These urchins, examined in the period from August 23 to September 11, yielded 79 cysts in which the larvae were dead, 60 cysts containing living larvae, 32 unencysted, dead larvae, and 16 unencysted, living larvae. The dead, encysted larvae were often partly disintegrated. Live larvae in cysts had extruded their stylets, but showed no evidence of development. The gonads were no larger than those of the cercaria and the ducts of the penetration glands were still visible. It is evident from these results that cercariae of the American species of *Zoögonus* will enter and encyst in sea-urchins and that they may live there for a time. But no development was observed and the finding of so many dead larvae, both free and encysted, indicates that *A. punctulata* is not a suitable host. It is probable, therefore, that these sea-urchins are not involved in the life cycle of the parasite.

The problem of specificity in host-parasite relations can be solved only by the experimental methods developed in studies on the life cycles of parasites. Formerly it was believed that different species of hosts harbored different parasites. In the case of trematodes, it is now known that a single parasitic species may infect a wide variety of hosts. *Allassostoma parvum* may infect frogs and turtles; *Zygocotyle lunata* may infect birds, rodents and ruminants; *Cryptocotyle lingua* may infect birds, rodents and carnivores; *Notocotylus urbanensis* may infect ducks and muskrats; *Fasciola hepatica* may infect cattle, pigs, rodents, the elephant, kangaroo and man; *Echinostoma revolutum* may infect various species of birds and mammals; *Psilostomum ondatrae* may infect the muskrat, duck, pigeon and canary. These examples, selected from a large list, represent five families and show that the possibility of multiple hosts is general. Furthermore, as a result of development in widely separated hosts, representatives of a single trematode species may manifest morphological differences which under other conditions might reasonably be regarded as specific. Specimens of *F. hepatica* from a guinea pig and others from a cow would hardly be assigned to the same species on the basis of morphology.

The digenetic trematodes manifest a comparable lack of specificity in their intermediate hosts. This is true particularly in cases involving a second intermediate host, often nothing more than a "transfer host" in which no development occurs. The condition is similar to that in *Fasciola* and *Zygocotyle*, where cercariae encyst on vegetation or other objects which are eaten by the final host. Even in the first intermediate

host, specificity may be far from rigid. For *Fasciola hepatica*, which has become cosmopolitan in distribution, snails belonging to the following genera may serve as first intermediate hosts: *Lymnaea, Galba, Bulinus, Physopsis, Physa, Stagnicola, Fossaria, Pseudosuccinea* and *Ampullaria.* In any particular region, one variety of snail is selected, but in different regions the species is different.

The data on *Zoögonus* are hard to interpret. In view of the lack of specificity in the life cycles of other trematodes, it is not impossible that a single species of *Zoögonus* employs different primary, secondary and definitive hosts on the two sides of the Atlantic ocean. In such event, the morphological variations are readily explained. On the other hand, the bionomic and morphological differences may represent valid specific criteria. This opinion is supported by cytological observations. According to Goldschmidt (1905), *Z. mirus* has 10 chromosomes, while Brooks (1930) found 12 chromosomes in the American form. At present there is no basis for a positive distinction between species of *Zoögonus* from the North Sea and the Mediterranean, but it appears probable that the European and American forms are specifically distinct. If this proves to be true, the American species is *Z. lasius* (Leidy, 1891) Stunkard, 1940.

Summary

Encysted metacercariae of *Zoögonus* are reported from the sea-urchin, *Psammechinus miliaris,* at Wimereux, France. Comparison with descriptions of other larval stages found at Roscoff and Marseilles indicates that all belong to the same species. Attempts to infect sea-urchins at Woods Hole with the American form of *Zoögonus* were only partially successful. Bionomic and morphological differences between the European and American representatives of *Zoögonus* are discussed. It appears probable that they belong to different species.

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FACTORS INFLUENCING MOULTING IN THE CRUSTACEAN, CRANGON ARMILLATUS

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Introduction

According to Darby (1938), *Crangon armillatus* exhibits diurnal moulting in which most moultings occur during the early afternoon. Although this is the warmest period of the day, he did not consider that there was any correlation with temperature. This investigation was undertaken in order to determine whether light or temperature, or both of these factors, are concerned with the diurnal moulting of this animal.

Crangon of different sizes were collected and placed in individual finger bowl culture dishes in the laboratory. The animals were fed abundantly on algae and the flesh of the spiny lobster, *Panulirus argus*. Some were kept on the table in the laboratory while others were placed in constant temperature incubators.

Diurnal Moulting

One hundred and thirty-six specimens of *Crangon armillatus*, varying in length from 10 to 39.5 mm., were selected so that there were approximately the same number in each size group, as shown in Table III. They were kept in individual finger bowls on the laboratory table where at midday the average light intensity was approximately 75 foot-candles. The experiment was begun on June 19 and continued until each animal had moulted twice with the exception of eight fatalities, for which other specimens were substituted. The data obtained in this study are shown in Table I.

This study confirms the existence of diurnal moulting in this animal under the conditions of this experiment. The great majority of the animals moulted between the hours of 10:00 A.M. and 5:00 P.M., with the largest number moulting between the hours of 1:00 and 2:00 P.M. No animals moulted between the hours of 9:00 P.M. and 7:00 A.M.

There is a diurnal rise and fall of air temperature at Tortugas which corresponds very closely with the diurnal moulting of *Crangon armillatus*. During the period of this experiment the average temperature in the

laboratory at 8:00 A.M. was 28.6° C., at 1:00 P.M. it was 31.9° C. and at 5:00 P.M. it was 29.4° C. The temperature in the laboratory at night fell to an average of approximately 27.8° C. Since the animals that were used in this experiment were kept in small dishes with a comparatively small amount of water they were, to a very large degree, subject to these temperature changes.

Under the conditions of this experiment the animals did not begin moulting until the temperature had risen to approximately 29° C. in the morning and ceased moulting when it fell to approximately this same temperature in the late afternoon or evening.

Crangon armillatus lives near low tide in small bays at Tortugas that are protected from strong wave action. At night and at high tide the temperature at this season is approximately 28° C. At low tide, at midday, the temperature in these bays often rises to 39° C., although the

TABLE I

Number of *Crangon armillatus* that moulted between the hours indicated when kept in individual culture dishes on the laboratory table.

7–8 A.M.	8–9 A.M.	9–10 A.M.	10–11 A.M.	11–12 A.M.	12–1 P.M.	1–2 P.M.	2–3 P.M.	3–4 P.M.	4–5 P.M.	5–6 P.M.	6–7 P.M.	7–8 P.M.	8–9 P.M.
1	6	6	21	26	27	55	33	31	25	18	16	5	2

average is four or five degrees lower. Thus in nature these animals are subject to much the same temperature changes as in the laboratory.

Effect of Keeping Animals at a Constant Temperature

This study was undertaken to ascertain if possible the effect of reversing the effect of daylight from daytime to night. Since it was discovered that the midday heat affected the incubators by raising the temperature above 30° C., when they were set at this temperature, a higher temperature was used.

Two incubators with thermostat control, which were set at 33° C., were used for this study. The inside of one of the incubators was illuminated from 8:00 P.M. to 8:00 A.M. by a Mazda lamp which cast a light of approximately 75 foot-candles on the animals, thereby reversing the relation between light and daytime. The inside of a second incubator was kept in total darkness both day and night in order to determine whether the light used in the first incubator had any effect on moulting.

Sixty-two *Crangon*, each in separate culture dishes, were kept in the illuminated incubator, and 57 were kept in the non-illuminated incubator.

TABLE II

Percentage of *Crangon armillatus* that moulted from 8:00 A.M. to 8:00 P.M., and from 8:00 P.M. to 8:00 A.M. when kept at 33° C. One group was kept in total darkness while the other was illuminated only at night.

	In total darkness	Illuminated with 75 f.c. at night
Moulted from 8:00 A.M. to 8:00 P.M.	54.4	51.6
Moulted from 8:00 P.M. to 8:00 A.M.	45.6	48.4

The number of moulted individuals were counted twice daily; at 8:00 A.M. to determine the number of individuals that moulted during the night and at 8:00 P.M. to determine the number that moulted during the daytime. The results obtained are shown in Table II.

Although the results above show that a few more animals moulted during the daytime than at night, moulting is not restricted to the daytime when *Crangon* are kept at a constant temperature. It further shows

TABLE III

Comparison of the average number of days between moults of *Crangon* that were kept in the laboratory at an average daily temperature of approximately 29.5° C. with those that were kept at a constant temperature of 33° C.

Size	10–15 mm.	15–20 mm.	20–25 mm.	25–30 mm.	30–35 mm.	35–40 mm.	Average days
At lab. temp. of approx. 29.5° C.	7	9.7	11.1	12.5	14.7	18	12.2
At 33° C.	5	7.8	9.2	9.6	11.5	14.1	9.5

that light of 75 foot-candles has little if any effect on the moulting of these animals. *Crangon* that are kept in culture dishes on the laboratory table moult only in the daytime or early evening whereas at a constant temperature they moult practically as often at night as in the daytime.

EFFECT OF AGE AND TEMPERATURE ON MOULTING RATE

Animals which were used in the preceding studies included 136 that were kept in the laboratory at laboratory temperature, and 119 which were kept at a constant temperature of 33° C. These were selected so that there were approximately the same number of animals in each of the six age groups shown in Table III. The average number of days between moulting periods for each size group is shown in the table. In this study, size was taken as a general criterion of age.

As shown in Table III the length of the period between moults was shortened, on an average, 2.7 days. By raising the average temperature

3.5° C. the moulting interval was decreased by 22.1 per cent. This is in agreement with Smith (1940), who showed that the length of the intermoult in young crayfish is directly dependent on temperature.

Moulting Rate of Non-seeded Females, Seeded Females and Seeded Females from Which Embryos Were Removed

For this study, 30 non-seeded females, 30 seeded females, and 30 seeded females from which the embryos were removed were placed in separate finger bowls on the laboratory table. All of the seeded females selected including those from which the embryos were removed were carrying very young embryos. Numbers were equally distributed among the three size groups shown in Table IV.

Table IV

Comparison of the average moulting interval in days of non-seeded females of different sizes with the interval between time of collecting and the next moulting period of seeded females bearing very young embryos, and with that of seeded females from which very young embryos were removed.

Condition of females	Size		
	20–25 mm.	25–30 mm.	30–35 mm.
Non-seeded females	11.4	12.1	14.3
Seeded females	16.1	15.7	18.4
Seeded females from which embryos were removed	11.2	10.3	12.9

In the above study it is impossible to state how much time elapsed between the last moulting period and ovulation in the case of the seeded females. The embryos that were attached to the seeded females were from one to three days old when the experiment began. If these seeded females moulted three or more days before ovulation, which seems probable, the period between moults of the seeded females would be approximately twice as long as that of non-seeded females. Moreover, when the embryos were removed from the seeded females the period between moults of these females was materially shortened. This indicates that there is something which inhibits moulting in seeded females.

In no instance did a seeded female moult while she was carrying embryos. However, all seeded females moulted within five days after shedding their embryos and twelve moulted within one day.

Discussion

The data presented in this paper indicate quite clearly that light of the intensity of 75 foot-candles has very little if any effect on moulting in *Crangon armillatus* and hence cannot be considered as an important factor in causing the diurnal moulting. On the other hand, the daily rise and fall in temperature is a very important factor in causing the diurnal moulting. Increase in temperature sets in operation the factors causing moulting, while a fall in temperature checks them.

From the above data it seems probable that at least two factors are concerned with moulting in *Crangon armillatus*. One, which causes moulting, manifests itself when the temperature in which the animal lives rises to or above approximately 29° C. The other, which inhibits moulting in seeded females, is apparently dependent upon the attachment of the embryos to the female. Moulting in insects, as shown by Wigglesworth (1934) and others is apparently caused by hormones. Brown and Cunningham (1939), Hanström (1939), Abramowitz and Abramowitz (1940), and Smith (1940) have shown the importance of a moult-inhibiting substance produced in the eye-stalk of crayfish and certain other crustacea.

Conclusions

1. *Crangon armillatus* exhibits diurnal moulting which begins in mid-forenoon, reaches its height at about 1:30 P.M. and ceases in later afternoon or early evening.

2. Light of 75 foot-candles has very little if any effect on moulting in these animals.

3. Temperature changes are very important in causing the diurnal moulting. Increase in temperature sets in operation the factors causing moulting while a fall in temperature checks them.

4. Animals kept at a constant temperature fail to exhibit diurnal moulting.

5. By raising the average daily temperature approximately 3.5° C. the moulting interval was decreased by 22.1 per cent.

6. Females carrying embryos do not moult even though the period of carrying embryos exceeds the normal period between moults.

7. At least two factors appear to be concerned with moulting. One, which is greatly influenced by temperature changes, causes moulting. The other, which inhibits moulting in seeded females, appears to be dependent upon the attachment of the embryos to the female.

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FEEDING MECHANISMS AND NUTRITION IN THREE SPECIES OF BRESSLAUA

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The question of food taking by protozoa has attracted considerable attention in the past and there have appeared numerous accounts of the various mechanisms employed, the type of food taken and the conditions of acidity and alkalinity during the digestive process. Regarding the last-mentioned observations there seems to be general agreement that, in the bacteria-feeding species at least, there is an acid-alkaline cycle from the time the food is ingested until the residue is defecated. Practically all of the observers employed some type or combination of types of indicator dyes, watching for the color changes which occur as the food is ingested, digested and the residue defecated. The most frequently used indicator has been neutral red because of the ease with which most protozoa take up this dye. Unfortunately, this indicator is useful only to detect shifts in hydrogen ion concentrations through a relatively small range.

We have examined the problem of feeding and acid-alkaline reactions in three species of the genus *Bresslaua*. These ciliates are carnivorous members of the family Colpodidae and one species, due to its peculiar feeding habits, offers exceptional opportunities for direct observations for long, uninterrupted periods of time.

In the ensuing account we will give a short description of the experimental organisms, an account of their feeding habits, some evidence for an acid-alkaline cycle during digestion and a brief account of food selectivity.

Material and Methods

The carnivorous ciliates were obtained from dry hay collected in Stuart, Florida. The same procedures of excystation and isolation were employed as were previously used in the case of most of the *Colpoda* material reported from this laboratory (Kidder and Claff, 1938; Kidder and Stuart, 1939; Burt, 1940).

For studies on the hydrogen ion concentration within the vacuoles

and the protoplasm various indicator dyes were used. These will be described in a later section. It was found expedient first to stain the food organisms (usually *Colpoda steinii*) and then to add a few *Bresslaua* to the culture. The culture was then placed in a moist chamber until the food organisms had all been ingested. As in the case of *Woodruffia metabolica* (Johnson and Evans, 1939; 1940), these carnivores formed resistant cysts after the food had become depleted. These were caused to excyst by the addition of fresh hay infusion. Food organisms were then added and the feeding process studied under a water immersion lens ($\times 40$). The dye brought into the protoplasm of the carnivores during the previous period of feeding was sufficient to allow us to gain an idea of the changes in acidity and alkalinity which took place during feeding, digestion and the subsequent defecation of residue.

For the study of food selectivity bacteria-free ciliates were necessary. The *Bresslaua* were freed of their associated microörganisms by the employment of our modification of the Parpart method of direct washing, using Syracuse watch glasses enclosed in cellophane bags (Kidder, Lilly and Claff, 1940). Because of the structural peculiarities of these ciliates it was found necessary to allow them to encyst and divide after the tenth wash in sterile hay infusion. Close watch was kept of the dividing ciliate so that the washing could be continued immediately after the emergence of the daughter organisms. Each of the two, four or more daughters was then washed individually through five or more changes of sterile medium and placed in tubes containing the food organism to be tested. Adequate bacteriological tests showed that the majority of the carnivores so treated were free of bacteria.

The food organisms tested will be discussed in a later section. They have all been mentioned in previous accounts from this laboratory.

Description of Bresslaua Kahl

The three members of this genus which we have studied resemble the various species of *Colpoda* in their general structure, mode of division within a cyst and permanent cyst formation. They all possess a macronucleus of the *Colpoda cucullus* type (Kidder and Claff, 1938; Burt, 1940) and a single micronucleus. The chief differences are found in the structure of the mouth, which has become modified and extended for the carnivorous mode of life. The following brief descriptions are given to add to the account of Kahl (1931) of *B. vorax* and to establish two new species.

Bresslaua vorax Kahl (*Fig. 1, A*).—This species is evenly rounded posteriorly, but the anterior end is compressed laterally. The left an-

terior side is depressed in such a way that the whole anterior end is twisted. This twisted appearance is seen best in an organism immediately after excystment. The size varies greatly depending upon the amount and kind of food taken. Freshly excysted ciliates range in length from 40 μ to 90 μ and in width from 25 μ to 50 μ. Ciliates which have fed on relatively large prey (such as *Glaucoma scintillans* or *Colpoda cucullus*) attain a size of 180 $\mu \times 100\ \mu$ or even larger.

The ciliary pattern, as seen after the silver technique of Klein or when treated with opal blue or nigrosin, resembles that of other members of the family. The peripheral cilia arise in pairs, as is true of most of the cilia among the members of the genus *Colpoda* (Taylor and Furgason, 1938; Burt, 1940). This is in contrast to the condition in *Woodruffiia metabolica* (Johnson and Larson, 1938) where the cilia are single. The cilia are relatively short and delicate. The ciliary rows originate from a short keel and extend over the general body surface as well as the right interior of the cytostomal cavity, converging in a field at the posterior end of the body.

The mouth is a large, cilia-lined cavity, open toward the ventral surface and the left side. On the roof of the mouth are folds or "rugae," roughly resembling those on the hard palate of mammals. On the floor of the mouth, which is somewhat raised, there is a row of membranelle-like structures, 40 to 45 in number. These beat in such a way as to create a strong current *out* of the mouth. At the back of the mouth there is a rather short, broad gullet directed posteriorly. It is on the brink of this gullet that the membranelles are located.

Bresslaua vorax exhibits activity when not actually feeding. It tends to remain on or near the bottom of the culture and to move in small circles. It comes in contact with the bottom so that the left side of the body, and therefore the mouth-opening, is up. Prey are swept into the mouth by strong currents. During the time the live prey is in the mouth until it has entered the food vacuole at the base of the gullet the movement of the carnivore is much reduced. This is due to a change in the beating of all the peripheral cilia and will be described in greater detail in the case of one of the other species. After the prey has been successfully trapped in the posterior food vacuole, movement is resumed.

Bresslaua insidiatrix sp. nov. (*Fig. 1, B*).—The general departures from the *Colpoda*-like structure which were described for *B. vorax* are accentuated in this species. The mouth opening is more extensive in relation to the size of the body and the twisting of the anterior end is somewhat greater. No "rugae" are present in the mouth. This species varies in size from 40 $\mu \times 25\ \mu$ when starved to 120 $\mu \times 90\ \mu$ when

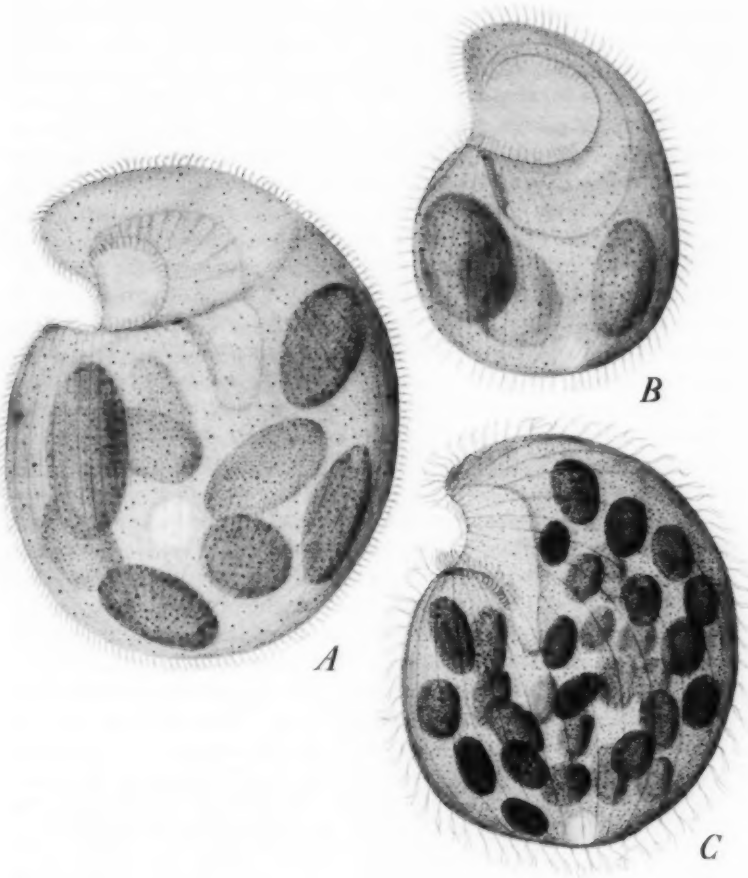

FIG. 1. All drawings were taken from life. × 460. *A. Bresslaua vorax.* The food inclusions are *Glaucoma scintillans. B. Bresslaua insidiatrix* sp. nov. during early stages of feeding on *Glaucoma scintillans. C. Bresslaua sicaria* sp. nov. after ingesting a number of *Colpoda steinii.*

ready to divide after active feeding. The general pattern of the peripheral ciliary lines is similar to that in *B. vorax*. The cilia originate in pairs and are very long and stiff. They are easily visible in life and stand out at nearly right angles to the body while the organism is at rest. There are 10 to 15 membranelle-like structures in the mouth located in the same relative position as those in *B. vorax*.

One of the most characteristic things about *Bresslaua insidiatrix* is its mode of feeding. It normally rests on the bottom of the culture dish with its right anterior end in contact with the substratum. It will remain for two to three hours in one spot, only occasionally pivoting slightly. During this time there is a strong current being directed into the very large mouth-opening and all small objects are drawn in. Inanimate objects are rapidly whirled toward the posterior border of the mouth and shot out by means of the out-going current created by the membranelles. Moving ciliates or flagellates, on the other hand, receive different treatment. Some mechanism within the mouth seems to be stimulated by ciliated or flagellated organisms and this appears to affect the whole neuromotor system. The peripheral cilia immediately lose their stiff, vibratile appearance and move slowly in waves (Fig. 2, *B, C*). The current going into the mouth slackens or disappears as does the outgoing current along the posterior border. The mouth-opening is contracted, forming an efficient barrier against the escape of the prey. The prey moves about freely in the mouth for from one to two minutes and gradually the posterior border of the mouth begins to form the prospective food vacuole. This vacuole forms well ahead of the prey and not under direct impact of it. The prey may partially enter the forming food vacuole and draw back into the mouth a number of times before it is finally trapped. Once well within the vacuole it begins to rotate and the vacuole closes off. The closure is effected by what appears to be a thin sheet of protoplasm originating from the region just posterior to the zone of membranelles and flowing across the vacuole opening from ventral to dorsal. At the instant the sheet of protoplasm fuses with the opposite side the prey is killed. This phenomenon will be discussed later in the section on hydrogen ion concentrations. The closure of the vacuole also sets off another reaction which immediately causes the peripheral cilia to resume their stiff, vibratile condition (Fig. 2, *D*).

Bresslaua insidiatrix appears to be the most highly specialized for a carnivorous habit of the three species observed by us. It feeds only on living ciliates and flagellates. Other bodies (cysts, amoebae, algae, yeast and detritus) do not evoke the "swallowing" response. That this evocation is largely physical is indicated by the following fact. In an excysting culture of *Colpoda steinii* it is common to see these small

ciliates rotating rapidly within the thin endocyst. These ciliates may be drawn into and swept out of the mouth of *B. insidiatrix* a number of times while the endocyst is still intact, but immediately the *Colpoda* escapes its cyst wall and is drawn into the mouth, it evokes the general responses noted above. In contrast to this, both *Bresslaua vorax* and the third species, yet to be described, are able to ingest certain types of non-moving microörganisms, but not all organisms ingested are adequate as food.

Bresslaua sicaria sp. nov. (*Fig. 1, C*).—This species shows a closer resemblance to the typical *Colpoda*-form than either of the above-mentioned species. The mouth opening is confined to the ventral surface and does not extend to the left side. The interior of the mouth cavity is similar in structure and relative size to that of *B. vorax,* but lacks "rugae." The zone of membranelle-like structures is composed of from 20 to 25 components and occupies the same general position as that in the preceding species. A well-formed gullet is present running posteriorly a short distance into the cell.

Bresslaua sicaria varies from 35 μ to 110 μ in length and from 23 μ to 92 μ in width depending upon its state of nutrition. The peripheral ciliary lines are less numerous than those of the other two species, but the general patterns are very similar. The cilia are long and wavy and originate in pairs.

Bresslaua sicaria, unlike the other two species, rarely comes to rest. It swims in a characteristic looping fashion and draws its prey into the mouth while swimming. There is a change in the ciliary motion during the act of swallowing resulting in general and violent movement of the whole organism. Immediately a food organism is caught the *Bresslaua* starts rotating rapidly on its lateral axis and continues the rotation until the prey enters the vacuole, when it resumes its swimming motion. The feeding reactions of this species are very difficult to observe because of its extreme activity.

The feeding habits of the three species described above are so characteristic that it is possible to identify each of them under very low magnifications. *Bresslaua vorax* and *B. insidiatrix* take their prey while they are in contact with the solid substratum, while *B. sicaria* feeds while swimming free in the medium. Of the first two, only *Bresslaua insidiatrix* remains motionless while waiting for its prey. Because of this characteristic, *B. insidiatrix* is an ideal carnivore to use in experiments and observations on feeding mechanisms.

The establishment of two new species of the genus *Bresslaua* seems to us to be justified because of the characteristics noted above (number of

ciliary rows, length and characteristics of cilia, shape and extent of cytostomal opening, feeding habits and food selectivity).

Food Vacuoles and Hydrogen Ion Concentration

After *Bresslaua insidiatrix* has fed on *Colpoda steinii* previously stained with a 1:12 million dilution of neutral red, it becomes highly colored by virtue of its food inclusions. After the food has been exhausted the carnivores form protective cysts. Many red food balls are still present in the encysted organisms. These food balls are defecated during or shortly following excystment (Fig. 2, *A, B*), leaving the ciliates nearly colorless. Under the water immersion lens it is possible to detect a number of neutral red stained granules in the endoplasm. Excystment with alkaline hay infusion imparts a slight yellowish tinge to the medium, but does not change the color of the endoplasmic granules. The small freshly excysted ciliates settle to the bottom of the culture dish and immediately begin feeding when numbers of *Colpoda steinii* are added with the excysting fluid (Fig. 2, *B*). The clearest observations are made during the capturing and killing of the first several ciliates.

As the prospective food vacuole forms its fluid contents become slightly pink (Fig. 2, *C*). This coloration deepens as the prey enters, but there appears to be no change in the motions of the prey at this time. At the instant that the food vacuole is closed off by the protoplasmic sheet there suddenly appear a large number of brilliant red granules or droplets in the protoplasm surrounding the vacuole (Fig. 2, *D*). The fluid surrounding the prey then becomes more deeply colored and simultaneously the prey is killed. The prey becomes motionless and the cilia stand out from the body. The fluid rapidly disappears from the vacuole and its lining comes to lie very close to the prey. The red granules in the cytoplasm rapidly fade out. There appears to be no indication that they enter the vacuole as has been described by Nirenstein (1905) for *Paramecium*. This is the first color-change to be noted. The reaction with neutral red shows that an acid condition is suddenly attained and that the hydrogen ion concentration is equal to or less than a pH of 6.8.

The above observations were repeated a number of times using a number of indicator dyes. None of them was quite as spectacular as the neutral red, either because they did not penetrate or because the colors were more difficult to see. Methyl red, methyl orange, brom cresol green, brom phenol blue, brom phenol purple, chlor phenol red, paradimethyl-amino-azobenzene (Töpfer's reagent), Congo red and benzene-azo-alpha-naphthylamine were used and of these methyl red was by far the best. Although not as brilliant as the neutral red reaction, all of

the phases appeared with this dye. The appearance of bright red granules with methyl red indicates that their acidity must be in the neighborhood of pH 4.2 or lower. Failure of blue coloration with Congo red indicates that the hydrogen ion concentration is probably not higher than pH 3.0.

It appears likely that the sudden death of the prey is the result of the release of an acid from the protoplasm of the carnivore into the vacuole. Töpfer's reagent failed to give positive results in this organism, although the dye penetrated well. Nirenstein (1905; 1925) had reported using this dye to detect the presence of mineral acid in the vacuole of *Paramecium*, as indicated by the appearance of a red color. No red coloration was obtained in *Bresslaua*. Just what type of acid is released is obscure.

Separate experiments show that the acidity indicated by the color changes with methyl red are compatible with the death points of the various types of prey. Thus, *Colpoda steinii* is killed almost instantly in a phosphate buffer of pH 3.8, while the *Bresslaua* is still alive after one hour at pH 3.4. *C. steinii* died only after long periods at pH 4.5 and above this value no death was observed. This experiment simply shows that *Bresslaua* is more resistant to high acidity than is *Colpoda* and lends support to the idea that the killing within the vacuole is a result of the release of acid. A similar conclusion regarding the function of acid was reached recently by Mast and Bowen (1940) in the case of *Vorticella*. Other food organisms which were tested were more resistant than *Colpoda*. *Euglena gracilis and Astasia klebsii* survived for a long time at pH 3.8 and this checks with the reactions of these two flagellates within the food vacuole of *Bresslaua*. After the protoplasmic sheet has closed over either of these organisms there elapses from two to ten minutes before euglenoid movement ceases.

Following the killing process the body of the *Colpoda* begins to move anteriorly due to the general cyclosis of the protoplasm of the *Bresslaua*. When the prey contains an indicator dye, such as neutral red, it is possible to follow the color changes occurring during the hour or two required for digestion. At first the prey is nearly colorless, but it rapidly becomes yellowish. This indicates a faintly alkaline reaction and corresponds to the situation found in *Paramecium* (Nirenstein, 1925) except that the vacuole has never been observed to swell. The yellow color remains for from 15 to 20 minutes and then gradually changes through orange to a bright cherry red (Fig. 2, *E*). By the time the prey has reached the red condition its general outline is lost and it has become a compact ball. A number of these balls later fuse and form the fecal mass which is extruded during or following the next encystment, either

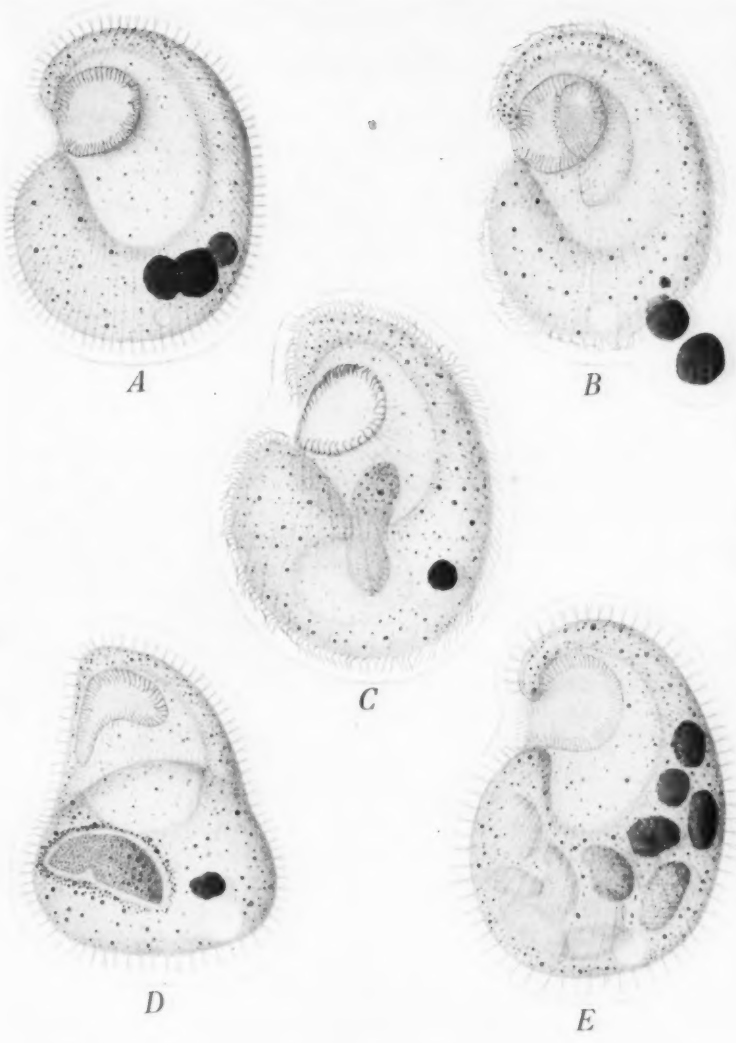

FIG. 2. *Bresslaua insidiatrix* showing the color changes with neutral red during feeding. *A*. Freshly excysted ciliate with old residue. Note the position of the cilia. *B*. Trapping of prey, *Colpoda steinii*, and defecation of residue. The cilia are bent and move in slow waves during this stage. *C*. Prey entering the prospective food vacuole, the fluid content of which is a faint pink. *D*. Food vacuole closed off from the mouth. At this stage the prey is instantly killed. Note the appearance of the cherry red granules in the cytoplasm surrounding the vacuole. The peripheral cilia of the carnivore have again assumed a stiff appearance. *E*. Carnivore after having ingested a number of ciliates. Note the color changes in the bodies of the prey as digestion proceeds.

from a division cyst or a protective cyst. Once released into the surrounding medium the fecal masses rapidly lose their red color, become pale yellow and disintegrate.

As mentioned previously, these observations are best made with *Bresslaua insidiatrix,* because of its feeding habits. As far as could be detected, the same general phenomena take place in the other two species. Certainly the color changes during digestion and defecation are the same, but the color changes accompanying killing, being of such short duration, could never be definitely established due to the constant movement of the carnivores.

Food Selectivity

The following account of the food selectivity is based on our observations of the three species of *Bresslaua* in the presence of a mixed flora of bacteria and in bacteria-free culture. While these observations do not represent a complete survey of the possibilities, they are presented in order to indicate the differences between the species and the possibilities for future work. In Table I we have listed the various food organisms which were used and have summarized the pertinent observations. It will be noted that ingestion does not invariably mean that the organism in question represents adequate food for growth. The various species of *Colpoda* supported growth in all three species of *Bresslaua* and these ciliates probably represent their natural food. The very nature of their protective cyst formation makes this assumption plausible. When dry hay is placed in spring water the various species of *Colpoda* excyst first, feed and multiply before the *Bresslaua* excyst. This means that in nature there would usually be a source of *Colpoda* at the right time.

Glaucoma scintillans was ingested by *Bresslaua vorax* and *B. insidiatrix,* while in the case of *B. sicaria* this was never observed. Thriving bacteria-free cultures of *B. vorax* and *B. insidiatrix* were maintained for a number of months with *G. scintillans* as food. In neither case, however, were normal protective cysts formed. After the food organisms had all been ingested the carnivores continued in the trophic condition for many days, getting smaller and smaller. Occasionally, in the case of *B. vorax,* they would round up and form temporary cysts (Johnson and Evans, 1940) from which they would spontaneously excyst within a few hours. This process might be repeated for days until finally all of the carnivores were dead. Serial transplants were always made while some food was still present. Eventually the various series declined in division rate and failed in transfer. The causes associated with this decline must receive further investigation.

TABLE I

Hay = sterilized hay infusion; *V* = *Bresslaua vorax*, *I* = *B. insidiatrix* sp. nov.; *S* = *B. sicaria* sp. nov.; (*N*) = non-sterile; (*S*) = sterile; (*S**) = no bacteria present but *Colpoda* were growing on *Stichococcus;* 1, 2, 3, 4, = relative growth where 4 is maximum observed. Where blanks occur in columns labeled "Ingestion" and "Growth" those organisms were not used.

Food Organism	Medium	Ingestion			Growth			Remarks		
		V	*I*	*S*	*V*	*I*	*S*	*V*	*I*	*S*
Colpoda cucullus....	Hay, H_2O	+	+	+	1	2	2	Large, normal (*N*)	Very large, normal (*N*)	Large, normal (*N*)
C. steinii.....	"	+	+	+	4	4	4	Medium, normal (*S*N*)	Medium, normal (*S*N*)	Medium, normal (*S*N*)
C. maupasi.....	"	+	+	+	4	2	3	Medium, normal (*N*)	Medium, normal (*N*)	Medium, normal (*N*)
C. aspera.....	"	+	+	+	1	4	1	Small, cysts with low viability (*N*)	Small, normal (*N*)	Small, cysts with low viability (*N*)
C. inflata.....	"	+	+	+	1	3	1	Small, cysts with low viability (*N*)	Small, normal (*N*)	Small, cysts with low viability (*N*)
Glaucoma scintillans	Yeast-Harris, H_2O	+	+	−	3	2	0	Very large, normal (*SN*)	Large, normal (*SN*)	(*SN*)
Tetrahymena geleii..	Hay, H_2O	+	+	+	1	0	0	Very large, abnormal. Cysts non-viable (*SN*)	Toxic (*SN*)	Toxic (*SN*)
Stichococcus bacillaris.....	"	+	−	+	3	0	0	Small, normal (*SN*)	(*SN*)	Encystment without division. (*SN*) Non-viable
Chlorella.....	"	+	−	+	2	0	0	Small, normal (*SN*)	(*SN*)	Encystment without division. (*N*) Non-viable
Euglena gracilis....	"	+	+		0	0		Encystment without division. Non-viable (*N*)	Encystment without division. (*N*) Non-viable	
Astasia klebsii.....	"	+	+		0	0		Encystment without division. Non-viable (*N*)	Encystment without division. (*N*) Non-viable	
Chilomonas paramecium.....	"	+	+		0	0		Encystment without division. (*SN*) Non-viable	Encystment without division. (*SN*) Non-viable	
Yeast (live).....	H_2O	+	−	+	3	0	3	Small, normal (*SN*)	(*SN*)	Small, normal (*N*)
Aerobacter.....	"	+	−	−	1	0	0	Small, normal (*N*)	(*N*)	(*N*)
Unknown bacteria..	"	+			3			Small, normal (*S*)		

Tetrahymena geleii was ingested by all three species but did not support continued growth in any case, although a few divisions of *Bresslaua vorax* usually resulted. *Tetrahymena* appears to be toxic to *B. insidiatrix,* for after a single organism had been ingested the carnivore would leave the bottom of the dish, swim rapidly for a few minutes and then round up and encyst. These cysts were never viable. *Bresslaua sicaria* behaved in a similar manner.

One other item worthy of note in these investigations on nutrition is the case of *Stichococcus bacillaris. Bresslaua vorax* readily ingests this alga and flourishing cultures result. Normal protective cysts are formed and may be collected, dried and stored for future use. By use of the glass plunger-sponge method (Kidder, 1941) any number of sterile carnivores may be kept on hand.

Discussion

While the work on the bacteria-free cultures has not progressed to a point where the nutritional requirements of the members of the genus *Bresslaua* can be stated definitely, a number of points of interest have come to light. One of the most interesting observations is the great difference in the food organisms as evidenced by the differences in nutritional quality between *Glaucoma scintillans, Tetrahymena geleii* and the various species of *Colpoda* for *Bresslaua. Colpoda* was utilized by all three species of *Bresslaua. Glaucoma* was utilized by *Bresslaua vorax* and *B. insidiatrix,* while *Tetrahymena* was utilized by only *B. vorax* and then the growth was poor and not transplantable. This is exactly the reverse of the situation with the carnivorous hypotrichs, *Stylonychia* and *Pleurotricha.* Lilly (1942) has shown that these ciliates will feed and reproduce on *Tetrahymena* but not on *Glaucoma* and *Colpoda.* It is becoming apparent that the exact nutritional requirements of carnivorous ciliates are delicately adjusted and this fact may be of use in the future for comparisons between food organisms.

Regarding our observations on the hydrogen ion changes during swallowing, killing, digestion and defecation, a few comparisons with reports of other workers may be noted. Prowazek (1898) described neutral red granules around the periphery of food vacuoles of *Paramecium.* He supposed that these granules might be the carriers of the digestive enzymes. An alkaline stage during the digestive process was not described. The work of Nirenstein (1905; 1925) was the most complete on this subject. He describes an initial acid phase in the newly-formed food vacuoles of *Paramecium,* the pH being equal to that of a 0.3 per cent solution of HCl. These vacuoles were much more acid than com-

parable ones in a number of other ciliates. After the initial acid stage the food vacuoles increased in volume and became alkaline. Nirenstein believed that digestion occurs only at this stage, the digestive enzymes being trypsin-like in nature. It had earlier been proposed by Hemmeter (1896) that the appearance of an acid phase was the response to living prey and that the acid served as a killing agent. This contention was denied by Métalnikow (1912) because he was unable to demonstrate any regularity in the acid production even in the event that living prey were ingested. In our work on *Bresslaua insidiatrix* the conclusion was reached that the initial acid production around the vacuole was stimulated by the closure of the vacuole and that it was probably this acid which caused the death of the prey. The prey did not become acid in its reaction, however, which may have been due to the combination of the acid with its proteins. Later, enough alkaline material was taken up to cause the protoplasm of the prey to give an alkaline reaction. This alkaline phase appears to be the phase of active digestion, indicating, therefore, that the enzymes involved are catheptic in nature. Before defecation the residue becomes acid, possibly due to the acidic properties of some of the products of digestion. The appearance of a final acid stage in the food vacuole in *Bresslaua* seems to differ from the condition in *Paramecium*. In the latter organism the residue remains alkaline (Shapiro, 1927).

Up to the present time most of the observations on the hydrogen ion concentration of food vacuoles have been confined to bacteria-feeding ciliates. It will be interesting to see if the conditions described above will be found in other carnivorous types.

Summary

1. Three species of *Bresslaua, B. vorax* Kahl, *B. insidiatrix* sp. nov. and *B. sicaria* sp. nov. are described.

2. These ciliates are carnivorous and feed on other small ciliates, members of the genus *Colpoda* being especially favorable as food.

3. Using indicator dyes it was found that the prey is killed simultaneously with a sudden release of an acid into the newly-formed food vacuole. The hydrogen ion concentration of the vacuole fluid was estimated to be between pH 3.0 and pH 4.2. This range includes the death point of various species of *Colpoda*. During digestion the protoplasm of the prey becomes alkaline. The undigested residue becomes acid before defecation.

4. Bacteria-free *Bresslaua* were tested with a number of food organisms and a preliminary survey of their food requirements recorded.

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SECRETION[1] OF INULIN, XYLOSE AND DYES AND ITS BEARING ON THE MANNER OF URINE-FORMATION BY THE KIDNEY OF THE CRAYFISH

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INTRODUCTION

The following data are the outcome of an attempt to find whether filtration occurs through the nephron of the crayfish. While they do not conclusively exclude filtration, they are sufficiently interesting to warrant presentation.

In contrast to the glomerular kidney of vertebrates, the aglomerular vertebrate kidney cannot eliminate glucose, even during hyperglycaemia and phloridzination (Marshall, 1930), and cannot eliminate xylose (Jolliffe, 1930), or inulin (Richards, Westfall, and Bott, 1934). Furthermore, there is little or no doubt that inulin is not secreted by nor passively resorbed through the vertebrate nephron (see Smith, 1937). Accordingly, it was presumed that the presence or absence of inulin in the urine of the crayfish (a classical freshwater invertebrate), after its injection into the haemocoele, would demonstrate whether filtration occurs.

It is here shown that although inulin and xylose do appear in the urine of the crayfish, they are, at least in part, actually secreted. It is therefore unnecessary to invoke filtration to explain the excretion of these carbohydrates. The ability of all parts of the nephron of this animal to secrete or accumulate one dye or another (see below) and of the coelomosac to secrete calcium (Maluf, 1941*a*) indicates that this nephron is mainly, if not entirely, a secretory organ.

The subject is *Cambarus clarkii,* which frequents the freshwater swamps of southern Louisiana.

[1] Throughout this paper, *secretion* implies the transport of a substance from a region of lower to one of higher diffusion potential for that substance. *Excretion* refers merely to the outward elimination of undesirable material, regardless of whether the latter is secreted or filtered.

This work was begun in the Department of Zoology, The Johns Hopkins University, while the author was Johnston Research Scholar. Many thanks are due to Prof. S. O. Mast for numerous kindnesses and appreciative criticism.

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METHODS

Inulin

Analysis.—The concentration of inulin in the blood and urine was measured, after acid-hydrolysis, by the Shaffer-Hartmann-Somogyi method (see Shaffer and Somogyi, 1933) using Shaffer-Hartmann reagent " 50 " and Somogyi's (1931) procedure for deproteinization of the serum. The technique was adapted to the small quantities used in this work as follows. Blood was taken by amputating a leg at the femur and allowing about 0.15 cc. to run into a small test-tube (9×75 mm.). Bleeding was instantly and permanently stopped by compressing the stump with the hot tips of a blunt forceps. The test-tube was stoppered and heated at 80° C. for about a minute or until the blood became opaque and was then cooled rapidly. The resulting solid was broken up with a fine glass-rod and the tube centrifuged for a few minutes. A sample of the supernatant liquid, usually about 75 cu.mm. was drawn into a fine calibrated pipette of the constricted type (Fig. 1, *A*). It was deproteinized by adding an equal volume of 7 per cent $CuSO_4 \cdot 5H_2O$ and another equal volume of 10 per cent $Na_2WO_4 \cdot 2H_2O$. Distilled water was added according to the required dilution (5 to 16 times), the same pipette being used in adding the water and reagents as that in taking the sample. After stoppering, shaking, and permitting to stand for at least 20 minutes, the tubes were centrifuged and 80 cu.mm. aliquots drawn for analysis. About 150 cu.mm. distilled water were added to increase the volume and then about 70 cu.mm. N H_2SO_4 for hydrolysis. The tubes were capped with glass-bulbs and heated in rapidly boiling water for 15 minutes. After cooling, a small drop of phenolphthalein was added. The solutions were neutralized with N KOH. If the color became too intense it was brought to pink with 0.1 N H_2SO_4; 0.161 cu.mm. of the Shaffer-Hartmann reagent was added and then a few drops of distilled water to augment the volume to about 1 cc. The test-tubes were shaken, capped with glass-bulbs, and heated without agitation in rapidly boiling water for 15 minutes. After cooling, the cap was removed only just before the contents of that tube were to be titrated and about 250 cu.mm. N H_2SO_4 introduced. The solid was completely dissolved with a glass-rod without undue agitation and the contents titrated with 0.01 N $Na_2S_2O_3$ until the color, due to the free I_2, became a very light yellow. About 35 cu.mm. of a 1 per cent aqueous solution of starch were added and the titration continued to the end-point. Titration was from a Linderstrøm-Lang-Keys microburette of 250 cu.mm. capacity, divided into cubic millimeters, and of uniform bore as shown by measurements of the length of a drop of mercury at all levels. The concentration of

inulin was ascertained by interpolation in a graph established from aqueous solutions containing a known quantity of the inulin (Pfanstiehl inulin, c.p.). Blanks and standards were run frequently. To obtain values with respect to the plasma, 5 per cent was deducted from the ascertained value, this being the approximate quantity of total solids in the whole blood of the crayfish and presumably close to the quantity which fell out by heating the blood at 80° C. The accuracy was within 5 per cent of the amount present.

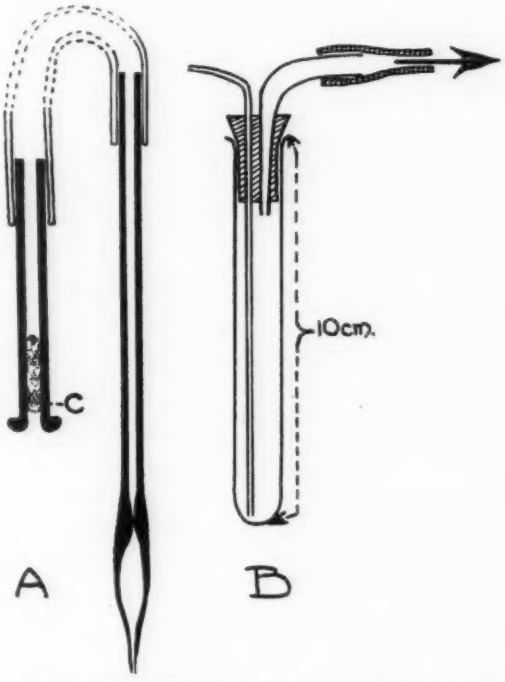

FIG. 1. *A*. Volumetric micro-pipette of the constricted type with mouth-piece and rubber tubing. *c*, cotton-plug. *B*. Apparatus for the collection of urine and emptying of bladders. Arrow indicates direction of suction.

The urine was treated in the same manner as the blood with the exceptions that heating and deproteinization were unnecessary, the urine being protein-free, and that, in the first 26 experiments, a correction for evaporation had to be applied (see below).

The preparation of inulin contained some impurity which, without being hydrolyzed, reduced Benedict's qualitative and which could not be

removed by yeast. The Pfanstiehl Company advised us that they had not been able to eliminate the impurity by repeated crystallization. The impurity was, however, negligible because within less than an hour after the injection of inulin, in the quantities used in this work (see Table II), the blood (whole or protein-free) was non-reducing unless subjected to acid-hydrolysis. Evidently the tissues removed the reducing substance rapidly. At no time could the urine reduce Benedict's reagent without preliminary acid-hydrolysis. Yeast-adsorption was therefore unnecessary in the analysis of inulin. Possibly because the animals were starved for a few days, the blood (whole or protein-free) of unsubjected animals was non-reducing even with preliminary acid-hydrolysis; the urine of these animals was invariably non-reducing. Crayfish can endure, without appreciable injury, starvation for four months at least (Brunow, 1911).

Inulin-clearance.—The renal clearance of a substance has a definite physiological meaning, being the virtual volume of blood cleared of that substance per unit time by the kidneys. It is expressed by $C = UV/P$, in which C is the clearance, P the concentration of the substance in the plasma, and V the rate of urinary flow. It is necessary to know the average concentration of that substance in the blood throughout the time that the urine to be analyzed is being formed.

Before an experiment, the crayfish was kept overnight fully submerged in running aerated freshwater. In measuring the inulin-clearance, the integument and branchial chambers were drained of moisture, the animal was weighed, and a fraction of a cc. of crayfish-saline [3] containing a given quantity of inulin injected slowly through the proximal abdominal venter and the wound cauterized. The amount of inulin injected was adjusted mainly by altering the concentration of the dissolved inulin in the saline because it was not desired to augment the blood-pressure by injecting a relatively large quantity of liquid (Table II). By thorough bleeding, the total quantity of blood in an average-sized *Cambarus clarkii* was found to be 6.6 per cent of the wet weight, which corresponds closely to the 6.7 of Herrmann (1931), who used the same method with *Potamobius astacus*.

After about 45 minutes the animal, including its branchial chambers, was drained of moisture and the anterior margins of the latter plugged with cotton-wool. The bladders were emptied by suction (about 12 mm. Hg) applied at the nephropores through the arrangement in Fig. 1, *B*.

[3] The saline was based on the most acceptable data on the concentration of inorganic electrolytes in the blood of the crayfish (see Maluf, 1940, for references) and was as follows (g./l.): NaCl, 7.81; $CaCl_2$, 1.31; $MgCl_2$, 0.82; KCl, 0.70; buffered at pH 7.6 by 0.5 cc. M/5 Na_2HPO_4/NaH_2PO_4. This assumes a Δ of about 0.66° C. (see Lienemann, 1938, and Schlatter, 1941).

When no urine could be obtained, firm bilateral digital pressure was applied to the integument lateral to the bladders (Maluf, 1941*a*) and suction again used until no further urine issued. The first sample of blood was taken immediately after and at what was considered zero time. The concentrations of inulin in blood from the pericardial sinus and from a leg were practically identical at that time, thus showing uniform distribution of the foreign material. The nephropores were cauterized to ensure a dry surface. "Ames Temporary [dental] Cement:

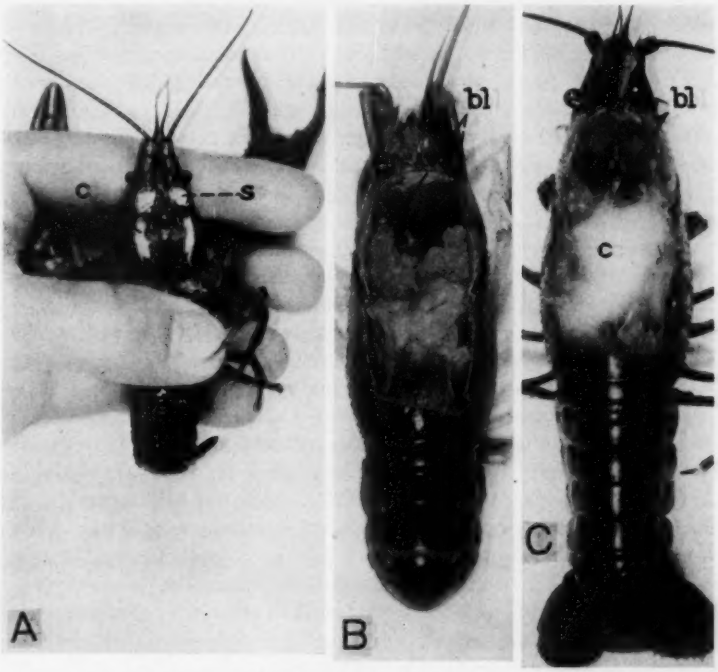

FIG. 2. *A*. Manner of handling the crayfish while the bladders are emptied and the nephropores are being sealed. *c*, cotton-plug in anterior margin of right branchial chamber; *s*, seal on the basal segment of the left antenna. *B*. Dorsal aspect of a crayfish with dorsal part of carapace and crop-gizzard removed, showing both bladders, *bl*, distended. *C*. Same as *B* but with viscera moved posteriorly and with wad of cotton, *c*, between viscera and bladders, *bl*.

hydraulic, non-irritant" was applied to the excretory eminence and basal segment of the antenna in two layers, under a magnification of 10.5 ×, by means of a forceps. The animal was handled as shown in Fig. 2, *A*. The cement should not extend beyond the basal antennal segment because movement of the antenna would be likely to crack the dried seal. Hard-

ening, which is due to the formation of zinc phosphate from zinc oxide and phosphoric acid, is rapid. Other cements were tried but were incomparably inferior. Ten minutes after completing the application the animal was fully immersed in freshwater and kept undisturbed.

Three or four blood-samples were taken through the experiment, which lasted 8 to 15 hours. Immediately after the last sample, the ventral nerve-cord was transected at the proximal level of the abdomen so as to prevent abrupt abdominal flexion, the chelipeds were amputated basally, and the dorsal surface of the carapace and the crop-gizzard, which is wedged over and between the bladders, were carefully removed. The distended bladders presented themselves conspicuously (Figs. 2, *B* and *C*, *bl*). The urine contained in the translucent bladders was crystal-clear and the kidneys could be seen beneath (Fig. 2, *C*). The viscera were then pushed back and a wad of absorbent cotton (Fig. 2, *C*, *c*) was applied over them to keep any fluid from flowing near the bladders. The urine was rapidly and completely collected by applying suction (about 12 cm. Hg) through the orifice of the arrangement shown in Fig. 1, *B* to the surface of each bladder. The animal was tipped on the side of collection with the head downward while this was done. The rate of urinary flow was thus accurately measured. The rapid collection obviated a correction for loss by evaporation. In the very few instances in which both bladders were not equally distended, the kidney corresponding to the lower rate of urinary flow was diminutive in size.

In the first 26 experiments on inulin-clearance the nephropores were not sealed because it was assumed that undisturbed animals with emptied bladders would not urinate appreciably during the interval. Urine, in these instances, was collected by suction from the nephropores and a correction applied for the fraction of water lost by evaporation. This is quite appreciable, and because previous investigators have not taken it into account the writer has no doubt that their values for the concentration of solutes in the urine of the crayfish are higher than the actual. The necessary corrections were obtained by aspirating, with the same pressure, a known quantity of distilled water, from the tip of a fine pipette of the constricted type (Fig. 1, *A*), into a test-tube of the same dimensions as that used for the collection of urine (Fig. 1, *B*). The resultant quantity of water, after light centrifugation of the test-tube for a few seconds, was measured by drawing it into a calibrated glass-tube. The loss in collecting 0.148 cc. in two minutes was 16.4 per cent and that in collecting 0.0739 cc. in five minutes was 35 per cent. The first correction was the one generally applied, as it was the writer's policy to collect the maximal quantity of urine in the minimum time with the above pressure. Generally an amount of 0.15 to 0.2 cc. could be

readily collected within two minutes. A successful and rapid collection depends to a great extent upon the aspirating tip. This should not have sharp edges but should be blunt and regular; its diameter should not be so large as to cover the entire excretory eminence. There is no doubt that the water lost was due entirely to evaporation. Scrupulous care was taken to prevent water-contamination of the urine, by draining the animal thoroughly, sucking water from the branchial chambers and rostral region, and plugging the anterior margins of the chambers with cotton-wool. The urine was not contaminated with blood, as was shown by negative biuret-, heat-, and H_2SO_4-tests, by an uninjured operculum, and by the fact that the concentration of inulin and dyes in the urine was considerably greater than in the blood (see below).

The rate of urinary flow was not measured in the first 26 experiments. It was assumed to be constant from one individual to another per unit weight, being determined, in a fully submerged animal, by the rate of diffusion of water into the body (Herrmann, 1931). Otherwise the rate of flow was measured by the above technique which necessitates sacrificing the animal at the end of the experiment. In ten experiments with inulin (Table I), the rate averaged 5.0 cc. per 100 grams per 24 hours. This is quite close to the average (= 5.2) of Lienemann (1938), who collected the urine by aspiration from nephropores which had been sealed, and was taken as the rate of flow for the animals in the first 26 experiments. The rate of urinary flow in the crayfish is low as compared with the frog (Forster, 1940) and freshwater turtle (Friedlich, Holman, and Forster, 1940), and even relative to that in birds and a terrestrial reptile (Marshall, 1932). This emphasizes the low permeability of the gills of the crayfish to water.

The inulin-clearances and U/P's obtained through the use of direct measurements of urinary flow with sealed nephropores (Figs. 6 and 7, *inulin;* solid circles) and from an average rate of flow with unsealed nephropores (Figs. 6 and 7, *inulin;* open circles) are quite comparable.

The average concentration of inulin in the blood through the experimental period was secured by averaging the interpolated values at the mid-period of each hour (see curves representing concentration-time, Fig. 3). Three or four blood-samples were sufficient to establish the shape of the curves. Furthermore, it was undesirable to take more blood than necessary.

Xylose

The analysis of xylose was identical with that of inulin except that acid-hydrolysis was omitted.

The nephropores were sealed and the rate of urinary flow measured directly (see above and Table I).

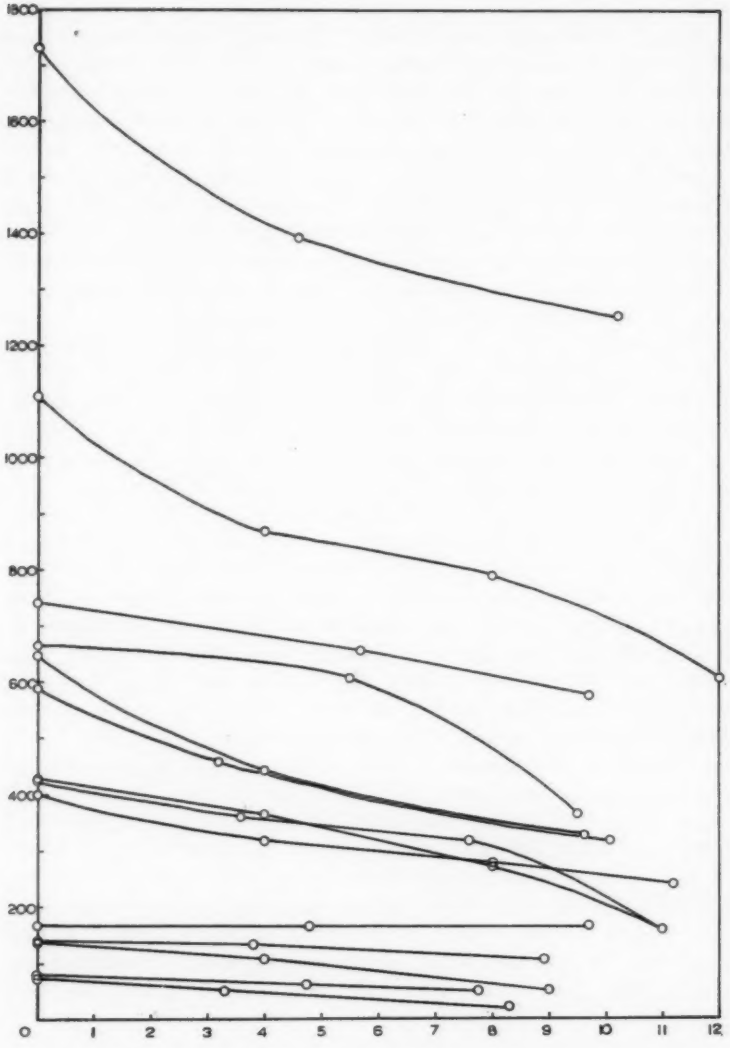

FIG. 3. The concentration of inulin in the blood in mg. per cent (ordinate) as a function of the time in hours (abscissa) during measurements of inulin-clearance.

Three blood-samples were sufficient (Fig. 4). The average concentration of xylose in the blood through the experimental period was calculated in the same way as for inulin.

Creatinine

Deproteinization of the serum was unnecessary because of the large dilution (about 26 ×). To 80 cu.mm. of serum or urine were added 2 cc. of distilled water. The tubes were capped and shaken and 1 cc. of the alkaline picrate was added to each. A Dubosque-type colorimeter with

TABLE I

Urinary flow in cc. per 100 grams per 24 hours.

No.	Male	Female	Material injected
1		3.4	0.3 cc. 10% inulin in crayfish-saline.
2		4.5	0.4 cc. 10% " " " "
3		3.3	1 cc. 20% " " " "
4		4.4	0.4 cc. 5% " " " "
5		6.1	0.4 cc. 5% " " " "
6	5.4		0.6 cc. 10% " " " "
7	6.4		0.6 cc. 10% " " " "
8		8.8	0.2 cc. 5% " " " "
9		4.4	0.2 cc. 5% " " " "
10	3.7		0.2 cc. 5% " " " "
11		5.4	1 cc. 30% xylose in ⅔ crayfish-saline.
12	7.2		1 cc. 30% " " " " " "
13	7.9		0.3 cc. 10% xylose in dist. water.
14		8.85	0.2 cc. 10% " " " "
15	7.1		0.5 cc. 10% " " " "
16	4.6		0.5 cc. 10% xylose in crayfish-saline.
17		2.9	0.5 cc. 5% creatinine in crayfish-saline.
18	4.6		0.5 cc. 5% " " " "
19		5.9	0.2 cc. 5% " " " "
20		5.7	0.2 cc. 5% " " " "
21	7.6		0.5 cc. 10% creatinine in dist. water.
22		8.9	0.5 cc. 10% " " " "
23	2.9		0.5 cc. 15% creatinine (somewhat toxic) in dist. water.
24		3.1	0.5 cc. 15% " " " " " "

1-cc. cups was used. The light was passed through a green filter. Standards were made each time as expected. There was never as much as a 50 per cent difference between the samples and the standards. The blood of unsubjected animals did not give a positive Jaffé reaction.

The blood-curves were almost straight lines (Fig. 5). The average concentration of creatinine in the blood through the experimental period was calculated in the same way as for inulin.

The nephropores were sealed and the rate of urinary flow measured directly (see above and Table I).

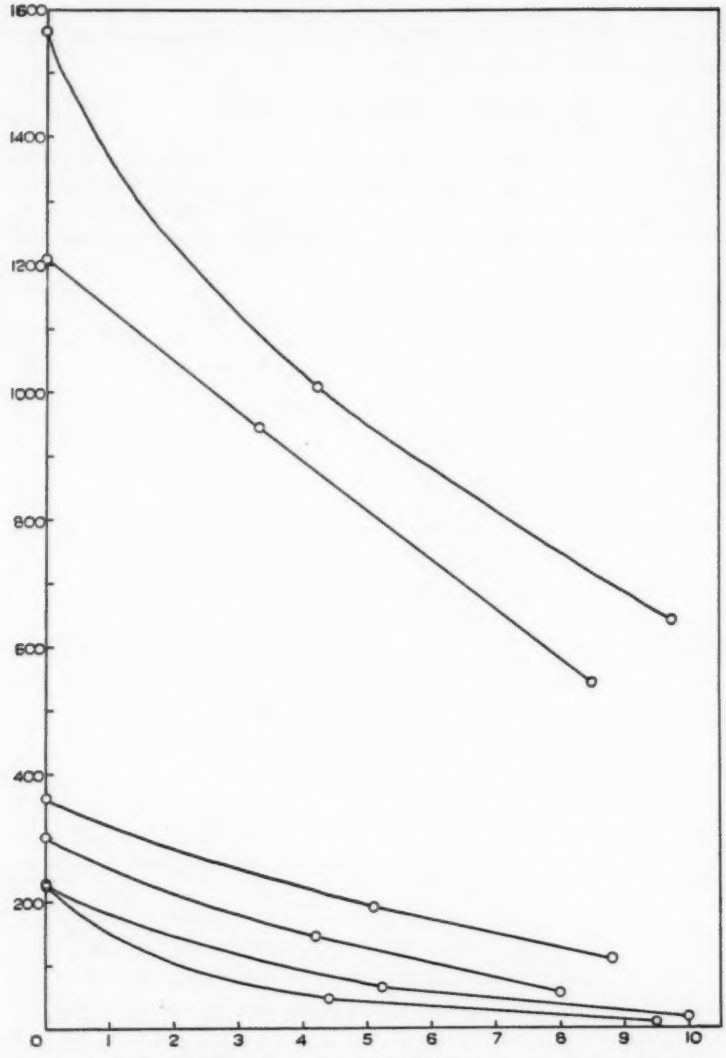

FIG. 4. The concentration of xylose in the blood in mg. per cent (ordinate) as a function of the time in hours (abscissa) during the measurements of xylose-clearance.

Results

Excretion of Inulin

In all the preliminary experiments, the injection of 0.2 cc. 5 per cent inulin in crayfish-saline resulted in a renal output of inulin. Thus, the urine, which was collected by suction from the nephropores, gave a positive result with Benedict's qualitative only after acid-hydrolysis. Before the introduction of inulin the urine was non-reducing even with acid-hydrolysis.

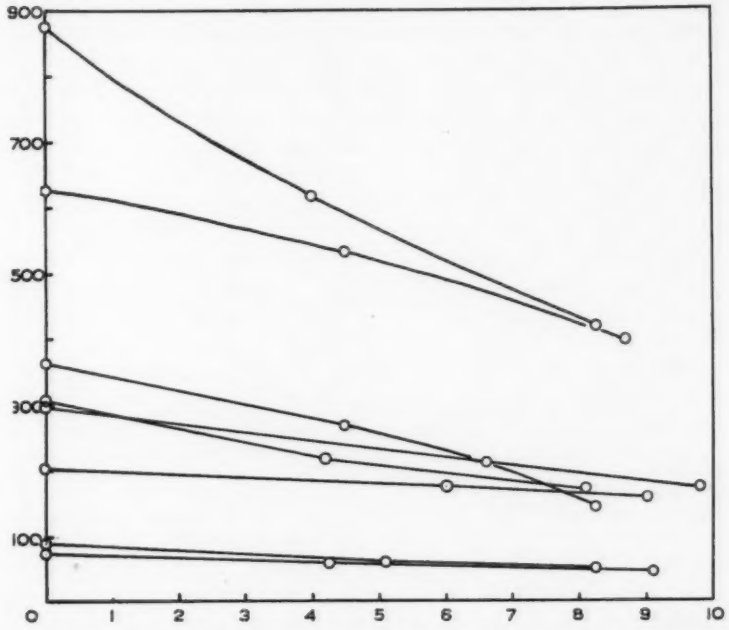

Fig. 5. The concentration of creatinine in the blood in mg. per cent (ordinate) as a function of the time in hours (abscissa) during the measurements of creatinine-clearance.

Similar results followed the injection of xylose or glucose. To guard against possible glucose-contamination in the sample of xylose, the urine was shaken for a few minutes with an equal quantity of a 20 per cent suspension of washed yeast in distilled water and centrifuged. Blanks, with only the yeast-centrifugate, were non-reducing. The glucose was given in high concentration (0.6 cc. 70 per cent per 40 grams) because the tissues tended to remove it from the blood.

Because glucose, xylose, and inulin are excreted by the kidney of the crayfish and not by the vertebrate aglomerular kidney, it at first seemed that filtration occurs in the former. This might imply that the hypotonicity of crayfish-urine is produced as in the Amphibia, namely, by the formation of a protein-free filtrate at the proximal end of the nephron and by subsequent resorption of relatively more salts than water by the tubule. On the other hand, other important data (see Discussion) contra-indicate filtration.

Because inulin is neither secreted by nor passively resorbed through the vertebrate nephron, the inulin-clearance in this phylum is an unvarying function of the concentration of inulin in the plasma. This is true

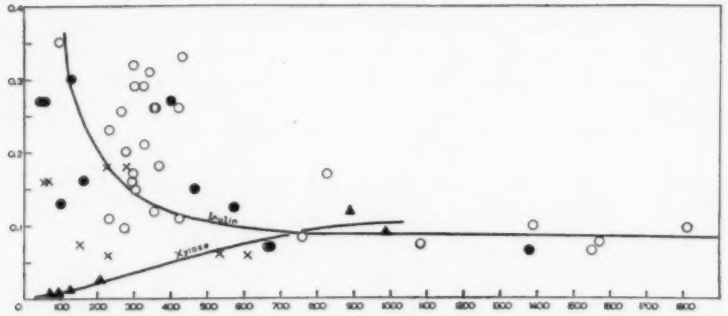

FIG. 6. The renal clearance of inulin, xylose, and creatinine in cc. per hour (ordinate) as a function of the concentration of these compounds in the plasma in mg. per cent (abscissa). *Solid circles,* inulin-clearances with direct measurement of urinary flow and nephropores sealed; *open circles,* inulin-clearances without direct measurement of urinary flow and nephropores not sealed; *triangles,* xylose-clearances with direct measurement of urinary flow and nephropores sealed; *crosses,* creatinine-clearances with direct measurement of urinary flow and nephropores sealed. Each point stands for a single separate animal.

even at the low plasma-concentrations (Miller, Alving, and Rubin, 1940). To find whether secretion can account for the marked occurrence of inulin in the urine of the crayfish, a study was made of the inulin-clearance at various levels of inulin in the plasma. Variation of the renal clearance with the plasma-concentration would demonstrate secretion. Parenthetically, even if the renal clearance of a substance does not vary with its plasma-concentration, secretion is not theoretically excluded (Shannon, 1938, 1939).

The actual inulin-clearance : plasma-inulin relationship (Fig. 6, *inulin*) demonstrates an outward secretion of inulin. The U/P : plasma-inulin curve (Fig. 7, *inulin*) is similar and the U/P's were above unity. Because the renal clearance is a product of the U/P and rate of urinary

flow, the approximate identity in the curves of Figs. 6 and 7 is equivalent to stating that the rate of urinary flow tends to be constant among different individuals.

It should be pointed out that the wet weight of both kidneys is normally a direct rectilinear function of the wet weight of the crayfish, at least in animals weighing between 10 and 50 grams. The relationship is expressed by $y = 0.0026x$, in which y is the mass of both kidneys and x the mass of the entire animal. Because the inulin-secreting mass of the kidney is probably a direct function of the total mass of the kidney, all animals should be approximately the same weight in an ideal set of experiments. In this investigation, because the lower plasma-concentrations were by no means confined to the larger animals (Table II), size, within the experimental range, cannot have been a determining factor in the inulin-clearance: plasma-inulin relationship. This is further brought out by the fact that the variation in the inulin-clearance with the concentration of inulin in the plasma, in crayfish which range between average and large size, is determined practically entirely by the U/P (Figs. 6 and 7, *inulin*) and not by the volume of urine excreted, which is greater in the larger animals although fairly constant per unit weight. In other words, the hourly differences in the absolute rate of urinary flow among individuals of somewhat different size are relatively small and inconsistent as compared with the variation of the U/P with the concentration of inulin in the plasma. Assuming a constant concentration of inulin in the plasma, the inulin-clearance ($= UV/P$) would doubtless vary with the mass of the kidney, but the U/P probably would not because the inulin-secreting mass of the kidney may bear a constant value with respect to the water-secreting mass. This implies that while the large kidney would secrete more inulin than the small one, it would also secrete proportionally more water.

The shape of the U/P: plasma-inulin curve indicates that the renal cells asymptotically become functionally saturated with inulin as the plasma-level of this compound rises. If filtration does not occur one would expect that, at *extremely* low concentrations of inulin in the plasma, the U/P's would be less than unity because there would be very little inulin available to the renal cells within a given interval of time. Apparently because of the relatively high avidity of the renal cells for inulin, it was not practicable to measure inulin-clearances at extremely low plasma-levels; sufficient urine was not formed before the blood was freed from inulin. With xylose, U/P's below unity occur even at moderate plasma-levels. It is possible that, at moderate concentrations, the kidneys secrete relatively more inulin than water and that the reverse is true for xylose.

Attempts were made to locate the site of inulin-secretion in the nephron by the colorimetric method of Alving, Rubin, and Miller (1939). About 0.8 cc. 10 per cent inulin in crayfish-saline were injected into

TABLE II

Excretion of Inulin

No.	Wgt. in g. and sex	Inulin		Urinary flow	Amt. of crayfish saline and conc. of inulin injected	Duration of experiment
		Plasma	Urine			
		mg. per cent	*mg. per cent*	*cc./hr.*		*hrs.*
1	42.7 ♀	433	1,590	0.088	0.6 cc. 10%	12.5
2	30.0 ♀	345	1,716	0.062	0.25 cc. 10%	11.5
3	27.8 ♀	355	1,600	0.058	0.27 cc. 10%	13.75
4	42.5 ♀	1,810	1,970	0.088	1 cc. 20%	14.25
5	34.2 ♂	1,390	1,974	0.072	1 cc. 20%	14.75
6	31.0 ♂	99	540	0.064	0.2 cc. 5%	13.5
7	25.7 ♂	234	468	0.054	0.2 cc. 5%	13.7
8	33.0 ♀	1,086	1,246	0.068	0.75 cc. 10%	12.5
9	42.7 ♂	235	578	0.090	0.4 cc. 5%	13
10	44.8 ♂	296	494	0.094	0.4 cc. 5%	12.6
11	33.4 ♂	758	883	0.070	0.5 cc. 10%	11.3
12	23.1 ♀	276	550	0.048	0.17 cc. 10%	11.5
13	51.1 ♂	304	825	0.106	0.38 cc. 10%	11
14	37.3 ♂	280	742	0.076	0.28 cc. 10%	13
15	51.7 ♂	356	841	0.108	0.45 cc. 10%	12
16	26.0 ♀	359	825	0.054	0.26 cc. 10%	12.3
17	28.0 ♂	299	858	0.058	0.28 cc. 10%	12.4
18	25.0 ♀	1,568	2,350	0.052	0.5 cc. 20%	11.6
19	23.0 ♀	1,549	2,130	0.048	0.5 cc. 20%	12
20	46.5 ♂	307	494	0.096	0.65 cc. 10%	14
21	48.5 ♂	425	462	0.10	0.68 cc. 10%	12.5
22	27.6 ♀	830	1,155	0.12	0.7 cc. 10%	12
23	21.7 ♀	330	718	0.094	0.2 cc. 10%	11
24	25.0 ♂	330	882	0.108	0.3 cc. 10%	10.6
25	20.4 ♀	300	1,090	0.088	0.4 cc. 10%	11.2
26	21.4 ♂	370	1,156	0.092	0.4 cc. 10%	11.2
27	28.0 ♀	670	1,180	0.040	0.3 cc. 10%	9.7
28	33.5 ♀	574	1,160	0.06	0.4 cc. 10%	9.5
29	30.3 ♀	1,380	2,220	0.042	1 cc. 20%	10.2
30	26.5 ♀	166	530	0.049	0.4 cc. 5%	9.8
31	29.5 ♂	131	520	0.075	0.4 cc. 5%	8.9
32	47.3 ♂	467	720	0.106	0.6 cc. 10%	9.7
33	32.0 ♂	424	1,250	0.089	0.6 cc. 10%	10.6
34	23.8 ♀	60	192	0.088	0.2 cc. 5%	7.75
35	29.5 ♀	102	240	0.054	0.2 cc. 5%	9
36	42.0 ♂	45	180	0.065	0.2 cc. 5%	8.3

medium-sized animals. After about three hours the kidneys were removed, rinsed in saline, and the coelomosac, tubule, and labyrinth teased apart. Approximately equal amounts of coelomosac, labyrinth, and tubule were put into separate small test-tubes. To each was added 1

cc. of the freshly prepared diphenylamine reagent. The tubes were capped and put into a boiling water bath for six minutes. The color which developed at the end of this time was evidently maximal. Unaided visual examination of the intensity of color did not indicate any differences in the amount of inulin present in the tubes. The intensity was determined solely by the mass of tissue used.

Regardless of whether a substance is removed from the blood by extrarenal tissues, the *renal* clearance of the substance will be a function of the concentration of that substance in the blood. It was nevertheless of interest to find if inulin can be hydrolyzed by the tissues of the cray-

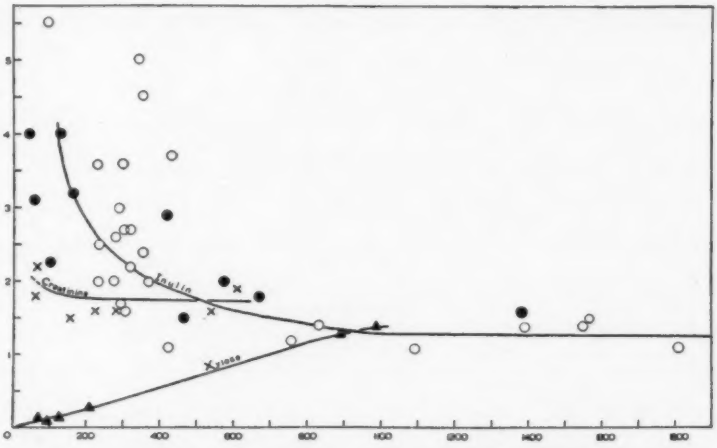

FIG. 7. The U/P ratio of inulin, xylose, and creatinine (ordinate) as a function of the concentration of these compounds in the plasma (abscissa). Notations identical with those in Fig. 6. Each point stands for a single separate animal; the same as those in Fig. 6.

fish. The kidneys and samples of the hepatopancreas, somatic muscles, and blood were frozen in solid carbon dioxide, thoroughly macerated, and extracted in a known quantity of saline. To aliquots of the centrifugates were added a solution of inulin and a small drop of xylol. The mixtures were analyzed for inulin immediately and after 13 hours at room temperature. The controls contained only a solution of inulin and the preservative. There was no change in the concentration of reducing carbohydrate, with or without acid-hydrolysis, in any tube. This indicates that, under the conditions of the experiments at least, inulin is not hydrolyzed by the tissues of the crayfish. Similar experiments showed a destruction of d-xylose in the following descending

order: hepatopancreas, kidneys, somatic muscles, blood. This may explain how the concentration of xylose in the blood falls more rapidly than that of inulin (Figs. 3 and 4) even though the renal xylose-clearances (see below) are lower than the inulin-clearances. Xylose may also diffuse out through the gills.

Excretion of d-Xylose

The xylose-clearance varies directly with the concentration of xylose in the plasma (Fig. 6, *xylose*) and at moderately low plasma-levels the U/P's are well below unity (Fig. 7, *xylose*). Assuming the occurrence of filtration and resorption, this relationship may be explained by an incapacity of the nephron to resorb xylose beyond a maximal rate; as a

TABLE III

Excretion of d-Xylose

No.	Wgt. in g. and sex	Xylose		Urinary flow	Remarks
		Plasma	Urine		
		mg. per cent	*mg. per cent*	*cc./hr.*	
1	29.6	988	1,370	0.067	1 cc. 30% xylose in ⅔ crayfish-saline; 9.75 hr. duration.
2	38.1	890	1,120	0.115	1 cc. 30% xylose in ⅔ crayfish-saline; 8.5 hr. duration.
3	31.5	89	10	0.10	0.3 cc. 10% xylose in dist. water; 10 hr. duration.
4	22.5	64	10	0.083	0.2 cc. 10% xylose in dist. water; 9.5 hr. duration.
5	33.6	210	61	0.10	0.5 cc. 10% xylose in dist. water; 8.8 hr. duration.
6	34.5	127	19	0.066	0.5 cc. 10% xylose in crayfish-saline; 8 hr. duration.

consequence, an increasing amount would "spill over" as the plasma-level is raised. Because at low plasma-concentrations the U/P is below unity (Fig. 7, *xylose*) the resorption would presumably be active, i.e. xylose would be inwardly secreted. There is a resemblance to the handling of glucose and other threshold-substances by the mammalian kidney.

On the other hand, the process can be readily explained, without resort to filtration, by assuming that both xylose and water are outwardly secreted and that, at low plasma-levels of xylose, the rate of secretion of water is relatively large compared with the secretion of xylose. At moderately high plasma-levels the xylose-clearance is nearly identical with the inulin-clearance (Fig. 6). In the experiments which necessitated the introduction of sufficient xylose to raise the average

plasma-concentration to about 1000 mg. per cent (see Table III), the animals became torpid soon after the injection but recovered completely within several minutes. It was therefore not considered within the scope of a physiological experiment to measure xylose-clearances at still higher plasma-levels. The injurious effects are probably osmotic. Inulin was not toxic even at the high concentrations.

For the same reason as with inulin, the xylose-clearance : plasma-xylose curve is practically identical with the U/P : plasma-xylose curve (Figs. 6 and 7, *xylose*).

TABLE IV

Excretion of Creatinine

No.	Wgt. in g. and sex	Creatinine		Urinary flow	Remarks
		Plasma	Urine		
		mg. per cent	*mg. per cent*	*cc./hr.*	
1	25.5 ♀	230	460	0.031	0.5 cc. 5% creatinine in crayfish-saline; 9.8 hr. duration.
2	26.5 ♂	155	240	0.051	0.5 cc. 5% creatinine in crayfish-saline; 9 hr. duration.
3	30.0 ♀	65	140	0.074	0.2 cc. 5% creatinine in crayfish-saline; 9.1 hr. duration.
4	39.0 ♀	60	105	0.093	0.2 cc. 5% creatinine in crayfish-saline; 8.25 hr. duration.
5	34.7 ♂	280	450	0.112	0.5 cc. 10% creatinine in dist. water; 8.25 hr. duration.
6	32.0 ♀	225	350	0.118	0.5 cc. 10% creatinine in dist. water; 8.1 hr. duration.
7	33.8 ♂	537	850	0.040	0.5 cc. 15% creatinine in dist. water; 8.7 hr. duration. (Somewhat toxic.)
8	25.5 ♀	610	1,150	0.033	0.5 cc. 15% creatinine in dist. water; 8.25 hr. duration. (Somewhat toxic.)

Excretion of Creatinine

Because the inulin- and creatinine-clearances are identical in certain vertebrates at all plasma-levels, it was desirable to compare the same clearances in the crayfish. The results were not elucidating and are presented here merely for record because it is believed that they are accurate (Figs. 6 and 7, *creatinine;* Table IV). Plasma-concentrations higher than 900 mg. per cent were definitely injurious if not fatal. The maximal ones on record are just within the threshold of toxicity, judging from the activity of the animal.

Excretion of Dyes

The initial objective of the experiments under this heading was to find if the nephron of the crayfish is capable of eliminating dyes which

the vertebrate aglomerular kidney is incapable of excreting. It was also desirable to study the capacities of the different parts of the nephron to secrete or accumulate various kinds of dyes.

The dyes were dissolved in crayfish-saline immediately before use. A description of the chemical composition of most of the dyes can be found in Conn's (1925) monograph.

Cyanol (DuPont).[4]—This is an aniline dye giving an intense blue in solution even when very dilute. Cyanol is not eliminated by the aglomerular vertebrate kidney if given in doses of the order of several mg. per kg. (Höber, 1930) but is slightly excreted when in quantities of 125–300 mg./kg. (Marshall and Grafflin, 1932).

Immediately after emptying the bladders, a fraction of a cc., containing a dose of about 1.7 mg./kg., was injected through the proximal abdominal venter. This colored the blood a vivid blue. Urine was collected after five hours and had to be diluted about tenfold to bring the intensity of color down to that of blood taken only one hour after the injection. Within five hours the blood lost all trace of blue. The experiment was repeated with similar results. As stated above, the concentration of foreign material, one hour after injection, is about equal in blood taken from the legs as in that from the pericardial sinus.

Other subjects were opened one to two hours after the injection. The viscera were rinsed with saline. Cyanol was not found in any organ other than the labyrinthic epithelium. The intensity of blue in the labyrinth not only greatly exceeded that of blood at the time but even that of blood taken only twenty minutes after the injection. The dye did not stain the bladder nor diffuse out from the contained urine even at a time, five or six hours after the injection, when it was absent from the blood.

The accumulation of cyanol in the labyrinth cannot be considered due to a resorption of water by the labyrinth, from a filtrate conceivably formed at the coelomosac, because: (1) The dye is greatly concentrated in the labyrinthic cells and yet not appreciably apparent in the more distal parts of the nephron; (2) the labyrinthic cells, even in "living" hanging-drop preparations, indicate a marked outwardly secretory activity as shown by the frequent presence of globules apparently being pinched off toward the lumen: the labyrinth therefore can scarcely be considered as a water-resorbing organ from a cytological standpoint; (3) the data indicate that the coelomosac is a secretory organelle (Maluf, 1941*a*, and below).

Ferrocyanide.—Iron salts, such as ferric ammonium citrate and sodium ferrocyanide, are not excreted by the aglomerular vertebrate ne-

[4] Kindly supplied me by Professor E. K. Marshall, Jr.

phron (Marshall and Grafflin, 1932) but are filtered through the glomerular nephron of vertebrates (see Smith, 1937).

Both bladders were emptied and 0.5 to 1.2 cc. of 2.4 per cent sodium ferrocyanide injected into animals weighing from 27 to 48 grams. The Prussian blue color was developed by adding a known quantity of Folin's (1929) ferric sulfate reagent to the Na_2WO_4–H_2SO_4 protein-free blood-centrifugate. At the end of five hours a scarcely appreciable quantity of urine could be collected, which gave a Prussian blue test. The ferric sulfate reagent produced an intense blue throughout the teased nephron; the color was more intense than that of the blood taken only 0.5 hour after the injection and seen through the same or greater depth. This experiment indicates that the kidney is capable of accumulating ferrocyanide but that the cells apparently become too poisoned to secrete urine. The hepatopancreas, muscles, and alimentary tract, rinsed free from blood and teased apart, gave no reaction.

Phenol Red.—Phenol red is secreted by the aglomerular teleost kidney (Marshall and Grafflin, 1932). The bladders of the crayfish were emptied immediately before the injection of the dye. The dose was 1 cc. of 34 mg. per cent phenol red into animals weighing about 30 grams. To develop the maximal intensity of color, both urine and blood were either exposed to NH_3 or received a known quantity of NH_4OH. The urine, collected five hours after the injection, had to be diluted over tenfold to reduce its intensity to that of blood taken 20 minutes after. On examining the kidneys in situ five hours after the injection, only the posterior part of the labyrinth had a reddish tinge. On adding a drop of 0.1 N NaOH to the nephron in crayfish-saline, the whole labyrinth became an intense red which was even deeper than that of blood taken as early as 0.5 hour after the injection. The labyrinth is thus capable of secreting phenol red and the pH of its cells is evidently about 7.0. Other tissues, including the coelomosac and nephric tubule, after being briefly rinsed from blood, showed no trace of phenol red.

The urine, as it issued from the nephropore, was a clear orange-red, not the purple-red of maximal intensity, and therefore has a pH of about 7.5.

Neutral Red.—In the three animals studied (dose: 1.2–1.8 cc. 80 mg. per cent per 30 grams) there was no indication of a concentration of this dye in the urine. The dye penetrated the labyrinth and tubule but the coelomosac did not show a trace of it. There seemed to be some accumulation in the hepatopancreas as the color was more intense in this organ (on adding a drop of acetic acid) than in the blood of equal depth. As the urine issues from the nephropore it is a light yellow and turns pink on the addition of acid. This shows that its pH is greater than 7.4.

It has already been noted that the phenol red experiments indicate a pH of about 7.5.

"Indigo Carmine."—Indigo carmine is composed of carmine blue and indigo disulfonate. The sample used had been passed by The Commission on Standardization of Biological Stains. It is long-known that indigo disulfonate is outwardly secreted by the vertebrate tubule. The dose was 0.7 cc. 80 mg. per cent per ca. 30 grams. Four hours after the injection the dye was markedly more concentrated in the bladder-contained urine than in blood even when collected only 25 minutes after the injection. On examination of the kidneys, no dye was found in the coelomosac or distal portion of the tubule. In one example concentrated dye was seen to leave the lumen of the proximal portion of the tubule upon application of pressure to the labyrinth, but there was no indication that the cells of the tubule take up the stain. The dye was concentrated in irregular patches in the labyrinth especially at the posterior end. There was no trace of it in the hepatopancreas and other tissues.

Congo Red.—Six-tenths of a cc. of 160 mg. per cent Congo red was injected into a 31-gram animal. Blood taken forty minutes later was a very light pink. The kidney was examined four hours after the injection; the coelomosac was a deep pink but the dye was absent from the rest of the nephron, and from the hepatopancreas, muscles, and gut.

"Basic Fuchsin" (aniline red; diamond fuchsin R.F.N.; magenta; passed by the C.S.B.S.).—The dose was 0.75 cc. 80 mg. per cent 30 grams. The animal was opened four hours after the injection. The stain had penetrated the muscles, hepatopancreas, coelomosac, nephric tubule, and other tissues. As compared with the blood, it was concentrated only in the labyrinth where it was a very intense purple. Soon after the injection the animals lay on their side in semi-torpor but recovered completely and removed all traces of dye from the blood.

Acid Fuchsin.—Eight-tenths of a cc. of 80 mg. per cent dye was injected into a 40-gram animal. The kidneys were examined after about 4.5 hours, at which time the dye was more concentrated in the bladders than in blood taken even 35 minutes after the injection. The labyrinth was a more intense pink than the blood seen through the same depth. The nephric tubule, coelomosac, hepatopancreas, muscles, and gut were not stained.

Methylene Blue (Passed by the C.S.B.S.).—Five-tenths of a cc. 50 mg. per cent were injected into a 22-gram animal. A blood-sample, taken after about two hours, was a very light blue. The animal was opened after about five hours. Methylene blue was concentrated only in the proximal portion of the tubule, where it existed as intracellular granules. After fixing the fresh kidney in 20 per cent formalin, the blue

concretions disappeared and the proximal portion of the tubule became a uniform blue. Evidently the intracellular granular condition depends upon an active process. The hepatopancreas and gills, but not the muscles, were merely stained. The experiment was repeated with identical results.

Colloidal Carbon.—Five-tenths of a cc. of "Higgins American India Ink: waterproof, black," diluted 6 × with crayfish-saline, was injected into a 20-gram animal. This was sufficient to give a very dark brown color to the blood. The animal was opened after four hours and the organs rinsed in situ with saline. The colloid had not penetrated any tissue. This is a functional demonstration of the absence of a nephrostome.

Discussion

The primary question is whether filtration occurs through the nephron of the crayfish. The paper of Bethe, von Holst, and Huf (1935), which appears to furnish positive evidence for filtration, should be read with care, especially as certain investigators have taken their results at face-value. Bethe et al. augmented the internal hydrostatic pressure of the crab, *Carcinus maenas*, by a vertical column of saline which communicated with the haemocoele. The aqueous column then sank in abrupt steps, indicating a fall in the internal pressure. They stated that this is evidently a physiological event because raising the hydrostatic pressure after death resulted in only a slight fall of the column which they attributed to an expansion of the soft membranes of the integument. They also pointed out that if the crab dies during the experiment the column of saline either does not fall or sinks very slowly. The animals, which were observed in air, were stated to have shown a loss of fluid from three sites: (1) the gill-chambers; (2) the mouth; and (3) the nephropores. These investigators noted that the fluid from the gill-chambers contained protein but was cell-free; the writer thinks that this fluid may have issued partly from the mucus-secreting glands. Above all, the authors explicitly remarked that, during the fall of the aqueous column, generally no loss of liquid by way of the nephropores could be observed. Their suggestion that the kidneys of the crab regulate the internal hydrostatic pressure per se, i.e. even when the osmotic pressure does not vary, is therefore unfounded. As noted above, augmentation of the blood-volume by about one-third apparently does not increase the rate of urinary flow.

By measuring the oncotic pressure of the blood and the haemocoelic pressure of crayfish, Picken (1936) indicated that filtration is apparently possible.

The writer is not aware of any facts which can be taken as positive evidence for the filtration-resorption theory or against the absence of filtration and the outward secretion of liquid. Analogy with the vertebrate nephron is inadequate. Furthermore, outward secretion of liquid is known to occur in aglomerular fish (Marshall, 1930; Bieter, 1931). The urine of the latter is, like that of the crayfish, hypotonic to the blood. It is unknown, however, whether the hypotonicity of the urine of aglomerular forms is due to an outward secretion of a hypotonic liquid or to the elimination of an iso- or even hypertonic liquid, in the proximal part of the nephron, followed by a resorption of salts. Owing to phylogenetical reasons (see Marshall, 1934), the latter method does not appear probable.

There are several facts which indicate that the nephron of the crayfish is primarily if not entirely an organ of outward secretion:

1. There is no tenuous syncytium such as the glomerular capsule of the vertebrate nephron (Maluf, 1939, 1941*a*).

2. Large calcareous concretions sometimes occur in the lumen of the coelomosac, the most proximal organelle of the nephron, thus indicating that the coelomosac can secrete calcium (Maluf, 1941*a*). The coelomosac is also capable of accumulating Congo red (see above).

3. Experimental cytological evidence indicates an outward secretion of water by the distal half of the tubule (Maluf, 1941*b*).

4. Histologically there is no doubt that the labyrinth secretes material outwardly (Maluf, 1939). The labyrinthic cells are capable of accumulating and outwardly secreting cyanol, phenol red, indigo carmine, basic fuchsin, and acid fuchsin (see above).

5. The cells of the proximal portion of the tubule can accumulate methylene blue (see above). All parts of the nephron are therefore capable of secreting or accumulating one dye or another.

6. Inulin is outwardly secreted (see above).

7. From a teleological viewpoint the coelomosac is evidently not a filtration-organelle (Maluf, 1941*a*).

8. Injecting into a moderate-sized crayfish 1 cc. of crayfish-saline, i.e. a volume about one-third that of the initial blood-volume, and thus very probably increasing the internal hydrostatic pressure, does not augment the rate of urinary flow (Table I).

The Malpighian tubule of insects, as a result of physiological and cytological evidence, probably should be considered as an entirely secretory nephron. The beautiful live preparations of Wigglesworth (1931*a*, *b*, *c*) show that the Malpighian tubule can excrete fluid, in an apparently normal way, even under conditions when the hydrostatic pressure is zero.

The ingenious experiments of Patton and Craig (1939) show that the Malpighian tubule can absorb various isotonic salines isosmotically even when the hydrostatic pressure must be zero (the saline rose into the capillary gauge up to 10 to 15 mm. admittedly by capillarity). They also state that hydrostatic pressure does not cause an increase in "filtration" rate. It is not apparent to the writer why Patton and Craig assumed that the isosmotic uptake of solution by the Malpighian tubules is due to filtration. It is known that the alimentary epithelium of vertebrates absorbs solutions isosmotically and, at the same time, absorbs, selectively, ions of a particular species.

Kowalevsky (1889), Cuénot (1895), and Bruntz (1904) studied the affinity of the crustacean nephron, in situ, for ammonium carminate, indigo carmine, and certain other dyes. They did not indicate, however, whether the dyes were concentrated by the nephron because they made no statements as to the relative intensity of dye in the blood and urine. Kowalevsky and Bruntz noted that ammonium carminate and litmus stain the coelomosac but not the rest of the nephron while indigo carmine stains the tubule and labyrinth. Because the coelomosac stained red with litmus, Kowalevsky concluded that this organelle has an acid reaction. He also observed that the coelomosac, and not the labyrinth, has an affinity for Congo red and methylene blue. Cuénot believed that the labyrinth of the crayfish, lobster, and crabs has a *strongly alkaline* reaction (italics his) because it "energetically decolorised acid fuchsin"; the color reappeared on macerating the kidney in acetic acid. He also noted that alizarin violet (an alkaline dye) retains its color instead of going into the orange-red phase. I have found, on the other hand, that the labyrinth is capable of concentrating acid fuchsin and that treating the nephron with acetic acid does not augment the intensity of color. The dye was more concentrated in the urine than in the blood.

The present observations with the pH indicators, phenol red and neutral red, show that the cytoplasmic pH of the labyrinthic cells is about 7.0 and that the pH of the bladder-contained urine is about 7.5. Because the labyrinth will take up an acid dye, such as indigo carmine, is no reason to believe that its cells are basic. The uptake of dyes during life is not equivalent to the affinity of fixed dead tissues for dyes. This is a distinction which Kowalevsky and Cuénot did not make.

The statements of Kowalevsky and Cuénot that the labyrinth is alkaline led the writer (1938) to suggest that the nitrogenous products of protein-catabolism are outwardly secreted by the labyrinth. The facts that the labyrinth is not alkaline and that the concentration of the N–P–N is markedly lower in the urine than in the blood (see Delaunay, 1927 and 1931, for the crabs *Maia squinado* and *Cancer pagurus;* the crayfish has

not been studied with this regard) have greatly weakened that supposition. It should be borne in mind that *M. squinado* and *C. pagurus* are marine crabs without nephric tubules and eliminate a urine isotonic with their blood. It is therefore unlikely that the N–P–N is subjected to dilution by an outward secretion of water.

Because the main nitrogenous excretory product of the Crustacea is a highly diffusible substance,—ammonia (Delaunay, 1927, 1931), it seems probable that this escapes largely through the gills. Although we possess data on the over-all rate of ammonia-output by the crayfish (*Potamobius astacus;* see Brunow, 1911), there is no statement in the literature concerning the concentration of ammonia in the urine; consequently the rate of output of ammonia by the renal route is unknown. Partly because the concentration of ammonia is practically the same in the urine as in the blood of the above-mentioned crabs, it is possible that the existence of ammonia in the urine is merely due to diffusion.

Summary

1. The techniques of measuring the rate of urinary flow and of collecting urine are described. The collection of urine from the nephropores by suction is a satisfactory procedure provided a correction is applied for the water lost by evaporation.

2. The techniques of collecting blood and of measuring renal clearances in the crayfish are described.

3. Raising the internal volume by one-third and therefore, presumably, augmenting the internal hydrostatic pressure, by the injection of 1 cc. of crayfish-saline, does not increase the rate of urinary flow.

4. Inulin and xylose will appear in the urine after being injected into the haemocoele. Glucose will occur in the urine provided enough is injected to permit its existence in the blood for a sufficient period.

5. The inulin-clearance and the U/P ratio of inulin vary inversely with the concentration of inulin in the blood. This demonstrates that inulin is secreted.

6. Inulin is not hydrolyzed by the hepatopancreas, kidneys, somatic muscles, or blood.

7. At low plasma-levels, the U/P ratios of xylose are very much below unity but rise above unity at high plasma-levels. This shows that xylose is either actively resorbed from a filtrate or is outwardly secreted but, with the low plasma-levels, at a relatively low rate compared with the secretion of water. The xylose-clearance : plasma-xylose curve is practically identical in shape with the U/P : plasma-xylose curve.

8. Although the renal clearances of xylose are much lower than the renal clearances of inulin, the plasma-concentration of the monosaccha-

ride falls more rapidly than that of the polysaccharide. This may be partly because the tissues can destroy xylose.

9. Only the labyrinthic cells can accumulate and outwardly secrete cyanol, phenol red, indigo carmine, basic fuchsin, and acid fuchsin. The coelomosac, but not the labyrinth or tubule, can accumulate Congo red. These dyes cannot accumulate in, and apparently do not penetrate into, other tissues of the body.

10. Only the cells of the proximal half of the tubule accumulate methylene blue.

11. Colloidal carbon does not enter the kidney; this is functional proof of the absence of a nephrostome.

12. The cytoplasmic pH of the labyrinthic cells is about 7; the pH of the bladder-contained urine is about 7.5.

13. The available facts (histological, chemical, physiological, and phylogenetical) indicate that the nephron of the crayfish is primarily if not entirely an organ of outward secretion.

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ACTIVATION OF CUMINGIA AND ARBACIA EGGS BY BIVALENT CATIONS[1]

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The activation of unfertilized eggs by isotonic salt solutions was first described by R. S. Lillie (1910, 1911*a*, 1911*b*). Since then, the activation by isotonic salt solutions of the eggs of seven marine invertebrates, belonging to three different orders, has been reported; *Pomatoceros* by Hörstadius (1923), *Asterias* by Dalcq (1924*a*, 1924*b*), *Phascolion* by Pasteels (1935), *Hydroides* by Pasteels (1935), *Barnea* by Dalcq (1928), *Thalessema* by Hobson (1928) and *Nereis* by Spek (1930).

The present work is a study of the effects of isotonic solutions of $CaCl_2$, $BaCl_2$, $SrCl_2$, $MgCl_2$, NaCl, KCl and LiCl, singly and in varying binary mixtures and proportions, on the eggs of *Cumingia tellinoides* and *Arbacia punctulata;* of the relative effectiveness of $CaCl_2$ in the activation of ovary eggs and shed eggs and of shed eggs that have been washed and shed eggs that have not been washed; and of the relative effectiveness of solutions of isotonic $CaCl_2$ which vary in pH. While its principal contribution is an extension of our knowledge of the effects of various isotonic salt solutions in the activation of eggs, it is hoped that it may illuminate further our understanding of the fundamental reaction or series of reactions which underlie the vital response of the cell.

Many careful investigators have shown that various types of stimulation cause an increase in permeability of the plasma membrane of various kinds of living material. However, Heilbrunn (1937) points out "There is one type of stimulation which can scarcely be conceived of as producing an increase in permeability. This is the stimulation produced by calcium salts. Students of permeability are quite unanimous in regarding the calcium ion as a permeability lowerer rather than a permeability increaser. Hence the action of calcium in producing stimulation cannot be explained on the basis of the permeability theory." Heilbrunn and his students have developed a colloid chemical theory of stimulation in which calcium plays the dominant rôle (see Heilbrunn, 1928; Heil-

[1] A thesis in zoölogy presented to the faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

brunn and R. A. Young, 1930; Heilbrunn and Daugherty, 1933; Heilbrunn and Mazia, 1936; Angerer, 1936; Mazia and Clark, 1936; Heilbrunn and Wilbur, 1937; Donnellon, 1938). This theory postulates the following series of changes: calcium is released from the cortex resulting in a liquefaction of the cortex; free calcium enters the interior of the cell; as the concentration of free calcium increases in the cell interior, a series of reactions is initiated which includes an initial decrease in viscosity followed by a characteristic clotting reaction. This series of reactions constitutes the vital response of the cell. My observations on the activating effect of bivalent cations appear to support the colloid chemical theory of stimulation as developed by Heilbrunn and his students.[2]

Material and Methods

In most of the experiments the same general procedure was employed. In any given comparison the eggs from one female were used. The eggs were shed into sea water, the supernatant fluid withdrawn and two drops of a dense suspension of eggs were quickly pipetted into dishes of experimental solutions previously prepared. No attempt was made to control the temperature of the experimental solutions. The temperature of the air was no higher than 26° C. at any time and was usually between 21° and 25°. In any given experiment the range was rarely more than 2° C.

Merck's C.P. chemicals were used in making the solutions. NaCl, KCl and LiCl were made up in 0.53 M concentration and $MgCl_2$, $BaCl_2$, $CaCl_2$ and $SrCl_2$ in 0.3 M concentration. These solutions are isotonic with sea water and the eggs do not shrink or swell in them. In studying the effects of various mixtures of isotonic salt solutions in the activation of eggs, the pH of the various solutions was adjusted, by the addition of 0.1 N HCl and 0.1 N NaOH, so as to lie in the range found experimentally to be most favorable for activation, i.e. pH 6.2 to 8.6 for *Cumingia* eggs and pH 8.8 to 9.0 for *Arbacia* eggs.

Eggs were exposed for varying periods of time to isotonic solutions of a single chloride or to mixtures of chlorides in varying proportions. The eggs were not transferred to sea water as is the usual procedure in experiments of this kind, inasmuch as a high percentage of cleavage could be obtained in the experimental solutions. In each experiment, hundreds of eggs were examined and 100 eggs were counted. The time factor is very important in determining the percentage of cleavage. At the end of

[2] This work was done at the Marine Biological Laboratory at Woods Hole during the summers of 1935, 1936 and 1937.

The problem was suggested by Dr. L. V. Heilbrunn. I wish to express my appreciation for his invaluable guidance and kind criticism during the course of this investigation.

The complete data are on file in the Library of the University of Pennsylvania.

a certain period, which is roughly three hours in the case of *Cumingia* eggs and five hours in the case of *Arbacia* eggs, there is no further increase in the percentage of cleavage and cell injury occurs a little later. It is desirable to count the percentage of activation at the end of this optimum period which varies with the solution, the pH and the temperature.

Conclusions concerning the effectiveness of a reagent in activating eggs are based on the percentage of cleavage. Although the first visible sign of activation of *Cumingia* eggs is the extrusion of polar bodies, it is difficult to make an accurate count of the percentage of eggs with polar bodies, for if the egg lies with the animal pole down, the polar bodies cannot be seen. While the first sign of activation when *Arbacia* eggs are inseminated is the elevation of the vitelline membrane, this reaction cannot be employed with isotonic solutions since they do not cause membrane elevation although they do cause the membrane to swell.

TABLE I

Experiments were performed to determine the relative effectiveness of barium, calcium and strontium solutions on the eggs of 33 individuals and tables were prepared of the percentage of cleavage and polar body formation and of the number of minutes elapsing before polar body formation.[4] The following results were obtained.

Activated by	Average time of pb formation	Average percentage cl	Average percentage pb
barium	11 min. 48 sec.	5.3	3.6
calcium	11 min. 27 sec.	35.4	26.1
strontium	10 min. 54 sec.	35.8	38.2
sperm	10 min. 6 sec.		

RESULTS

Cumingia

Effect of 0.3 M $CaCl_2$.—When unfertilized *Cumingia* eggs are placed in 0.3 M $CaCl_2$, at any pH between 6.0 and 8.6, the first polar body is extruded in from 5 to 12 minutes and the first cleavage is completed in from 40 to 60 minutes. This is approximately the same as the time of polar body formation and of cleavage in eggs activated by sperm. The percentage of cleavage at the end of several hours varies widely among the eggs of different individuals. In some individuals 100 per cent of the eggs undergo apparently normal activation. They continue to cleave for several hours, reach the 8–16 cell stage and appear to be healthy and normal. After several hours, however, the blastomeres pinch in and fall apart.[2] Polar body formation is extremely irregular, but there appears

to be an inverse relationship between the percentage of polar body formation and the percentage of cleavage (compare Morris, 1917).

The result of experiments in which eggs were exposed to solutions of $CaCl_2$ which vary in pH from 3.6 to 9.0 is shown in Table I. The various solutions from pH 6.0 to 8.6 are equally effective in inducing activation of the eggs of most individuals. The pH of the solutions appears to have more effect on the percentage of cleavage than on the percentage of polar body formation, polar body formation proceeding at pH 9.0 while the percentage of cleavage decreases above pH 8.6.[3] Both are almost completely inhibited at pH 4.1. (See Table II.) While the

TABLE II

Effect of pH on Activation of Cumingia Eggs by Isotonic Calcium Chloride

No. of exp.	1	2	3	4	5	6	7	8	9	10
Time of expos. Hrs.	4	4	4	6	6	7	4	4	6	7
Min.	20	55	15	20	20	15	20	55	55	15
Temp. °C.	21.3	21.3	21.3	20.0	20.0	20.0	21.3	21.3	20.0	20.0
	pb cl	pb cl	pb cl	pb cl	pb cl	pb cl	pb cl	pb cl	pb cl	pb cl
pH										
4.1	0 0	3 1	0 0			0 0	0 0	0 0		
4.6				60 5	32 0	19 0	0 0		13 0	40 0
4.9	55 2	34 18	21 12				14 38	9 27		
5.8				13 70	40 13	60 2		7 70	18 1	35 2
6.1	13 45	16 51	20 19				16 50			
6.2				2 98	6 98	4 94			30 5	8 10
7.1				2 98	6 88	6 91			25 5	2 14
7.6	22 45	10 63	30 32	2 98	5 90	9 89	16 55	8 66	56 3	3 15
8.2	18 49	14 59	11 54	32 92			14 59	4 65		
8.3				2 96	6 92	6 90			52 0	6 26
8.6	19 60	18 50	20 55	13 87	11 88	10 77	19 60	10 50	35 1	12 27
9.0	17 33	24 22	6 52	34 22	27 67	27 67	17 33	12 55	20 3	13 18
sw	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0

strontium solution is somewhat more effective than the calcium solution, the former causes the blastomeres to separate in less time. It is difficult to make accurate counts of the percentage of cleavage when blastomeres separate.

Effect of Mixtures of Calcium Chloride and Some Monovalent Cations.—The result of experiments in which eggs were exposed to mixtures of potassium chloride, sodium chloride or sea water and calcium chloride in various proportions is shown in Fig. 1. The degree of activation decreases rapidly as the proportion of potassium increases up to a ratio of 1–16 and then remains fairly constant. It may be concluded that there is an antagonism of calcium by potassium between ratios 1–64

[3] This work is not a study in artificial parthenogenesis and no attempt was made to develop procedures for securing later stages of development.

and 1–16 since the percentage of activation decreases too rapidly to be due to the dilution of calcium ions by potassium ions. Sodium has only a slight inhibiting influence on the activating effect of calcium from ratio 1–64 to ratio 1–4, but inhibition increases markedly above an average ratio of 1–4. The slight decrease in the percentage of activation up to an average ratio of 1–4 is probably due to the dilution of the calcium

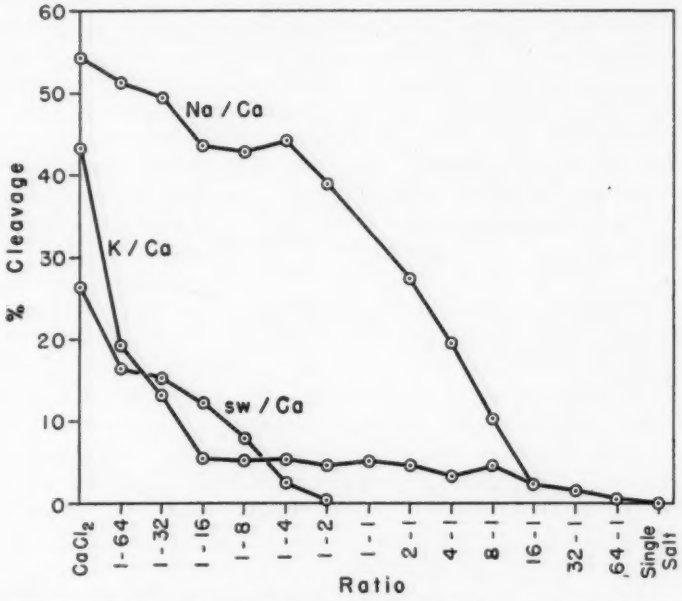

FIG. 1. Action on *Cumingia* eggs of isotonic solutions of some monovalent cations and of $CaCl_2$ singly and in various mixtures and proportions.

K/Ca—average of ten experiments.
Na/Ca—average of sixteen experiments.
sea water/Ca—average of five experiments.

solution, but there is an antagonism of calcium by sodium above this ratio. Sea water has an inhibiting effect on the activating effect of calcium beginning in a ratio of 1–64 and increasing as the proportion of sea water increases, with complete inhibition in most cases at a ratio of about 1–2. The inhibiting effect of sea water is no doubt due to the monovalent cations.

Effect of Mixtures of Calcium Chloride and of Some Bivalent Cations.—The result of experiments in which eggs were exposed to mixtures of strontium, barium or magnesium and calcium in various propor-

tions is shown in Fig. 2. Strontium is somewhat more effective than calcium in inducing cleavage in *Cumingia* eggs but causes the polar bodies to be extruded far from the cell surface and the blastomeres to separate in a short time. Strontium inhibits slightly the activating effect of calcium while calcium inhibits somewhat the activating effect of strontium. Solutions in which the Sr/Ca ratios are from 1–1 to about 64–1 are more injurious than the single salt solutions and it is difficult to make an accu-

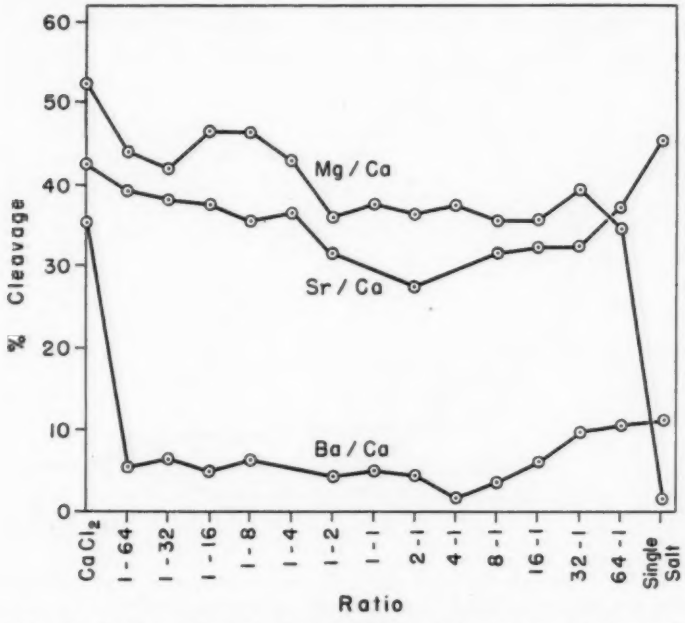

FIG. 2. Action on *Cumingia* eggs of isotonic solutions of some bivalent cations singly and in various mixtures and proportions.

Sr/Ca—average of seventeen experiments.
Ba/Ca—average of twelve experiments.
Mg/Ca—average of thirteen experiments.

rate count of the percentage of cleavage between these ratios. Barium has a slight activating effect on *Cumingia* eggs. It may be concluded that there is an antagonism of calcium by barium as the latter produces a marked inhibition of the activating effect of calcium, beginning in a ratio of 1–64. The percentage of activation resulting from exposing eggs to isotonic $MgCl_2$ is negligible. Magnesium inhibits very slightly the activating effect of calcium even in a ratio of 64–1. Inasmuch as magnesium has no activating effect on *Cumingia* eggs, it might be ex-

pected that as the dilution of the calcium solution by magnesium increases, the percentage of activation would decrease. It is interesting and noteworthy that this is not the case. Even in an Mg/Ca ratio of 64–1 there is a high percentage of cleavage.

In summary, monovalent cations are not effective in activating the eggs of *Cumingia* and they inhibit the activating effect of calcium, the effect increasing as the K/Ca, Na/Ca, sea/Ca ratios increase. The bivalent cations, with the exception of magnesium, are able to activate *Cumingia* eggs and in certain combinations and proportions, mutually inhibit activation. There is considerable variation in the behavior of different lots of eggs, but it is a variation in magnitude rather than in kind. The results of experiments performed on the eggs of a single individual (see original manuscript) are more interesting than the average of the results of many experiments as presented in this paper, because the former presents a more characteristic picture of the behavior of marine eggs.

Arbacia

Effect of 0.3 M $CaCl_2$.—When unfertilized *Arbacia* eggs were placed in 0.3 M $CaCl_2$, at any pH between 8.0 and 8.5, a certain percentage (rarely more than 25 per cent) of the eggs of most individuals undergo cleavage. It is difficult to make reliable counts of the percentage of activation in *Arbacia* eggs inasmuch as cells that have undergone cleavage usually occur in groups. The time required for maximum percentage of cleavage is from 7 to 10 hours or considerably longer than the time of cleavage in eggs activated by sperm. In a study of the eggs of 40 individuals the percentage of cytolysis was found to be high (33.5 per cent) if the eggs were aged for about 11 hours before being placed in the calcium solution.

A comparative study was made of the percentage of cleavage when the eggs were obtained in various ways. In some instances a fragment of ovary was placed directly in the solution to be tested. The exuding eggs are called ovary eggs. If such exuded eggs were washed in sea water they are called washed eggs. Shed eggs were obtained in the usual manner. Experiments were performed on the eggs of about 60 individuals and tables were prepared on the comparative percentage of cleavage. These tables are elaborate and it was thought unwise to attempt their publication (see original paper). The following results were obtained:

	Average percentage of cleavage
Ovary eggs	11.8
Shed eggs	22.8
Shed eggs	23.8
Washed shed eggs	30.9

TABLE III

Effect of pH on Activation of Arbacia Eggs by Isotonic Calcium Chloride

No. of exp.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Time of expos. Hrs.	7	7	7	7	8	8	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	6	7	7	7	5	5	5	5	5
Min.	21	21	21	21	45	15	15	15	15	15	15	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	5	20	20	20	7	7	7	7	7
Temp. °C.	22	22	22	22	21	21	22	22	22	22	22	22	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	25	25	25	25	25	25	25	25
	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl	cl
pH																																			
3.8	0	0	0	0																															
3.9					0	0																													
4.2	0	0	0	0	0	0																													
6.0	0	0	0	0																															
6.0					0	0																													
6.5														0	0	0	0	0	0	0	0	0	0	0	0	0	0								
6.9							1	0	0	1	0	0	0																							
7.0	0	0	0	0	0	0																													
7.2																												1	0	0	0	0	0	0	0
7.3							0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								
8.0					2	0	0	0	3	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	1	0	0	0							
8.1																															0	0	0	2	2
8.2																												1	1	0					
8.3					10	0																													
8.4							0	0	5	1	10	12	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0				5	7	8	6	0
8.6	35	2	10	19	0	0	5	2																				23	33	2					
8.8							6	18	6	21	30	8	15	34	0	0	0	0	0	0	0										3	7	9	2	2
9.0							5	20	25	7	18	15	36	70	21	33	12	90	15	51	39	55	39	47	12	46	24	23	5	4					
9.4																														13	18	10	22	15	18
9.6	18	30	45	25	15	10																													
sw	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

There is a higher percentage of cleavage in shed eggs than in ovary eggs and a slightly higher percentage of cleavage in washed shed eggs than in shed eggs.

Table III shows that when the unfertilized eggs of one individual are placed in solutions of $CaCl_2$ in which the pHs vary from 3.8 to 9.6, for from 5 to 9 hours, the percentage of cleavage is low below pH 8.0 and is only slightly higher at pH 8.5 while the highest percentage of cleavage takes place at about pH 9.0. Cleavage takes place in the short-

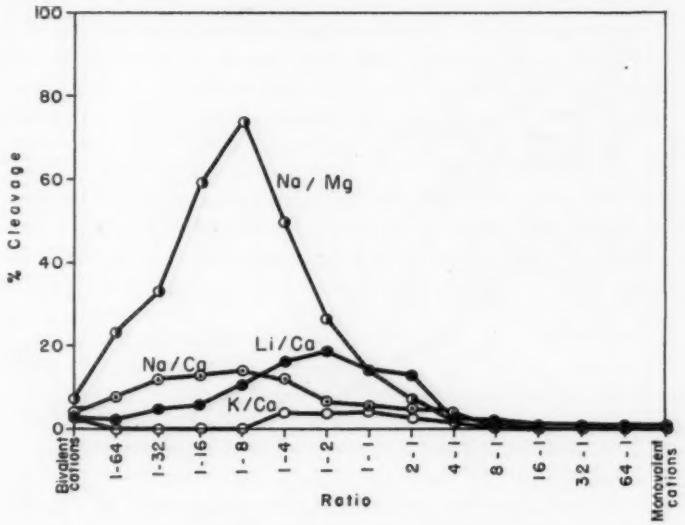

FIG. 3. Action on *Arbacia* eggs of isotonic solutions of some monovalent and bivalent cations singly and in various mixtures and proportions.

K/Ca—average of fourteen experiments.
Na/Ca—average of nine experiments.
Li/Ca—average of eight experiments.
Na/Mg—average of three experiments.

est time at pH 9.2 but the eggs soon undergo cytolysis. High alkalinity also seems to cause nuclear division without cytoplasmic cleavage in a large percentage of eggs. In many cases there is little and sometimes no cleavage in solutions of which the pH is about 8.8 while there may be a high percentage of cleavage in solutions of which the pH is about 9.0. *Arbacia* eggs are not activated by alkaline sea water.

Effect of Mixtures of Calcium Chloride and Some Monovalent Cations.—The result of experiments in which eggs were exposed to solutions of potassium, sodium or lithium and calcium chloride in various

proportions and of sodium and magnesium chloride in various proportions is shown in Fig. 3. There is a gradual increase in the percentage of activation as the proportion of potassium increases up to a ratio of about 1–2 while above this ratio there is a decrease with activation ceasing at a ratio of about 8–1. From ratios 16–1 to 64–1 there are many eggs in which the nucleus has undergone several divisions and in which

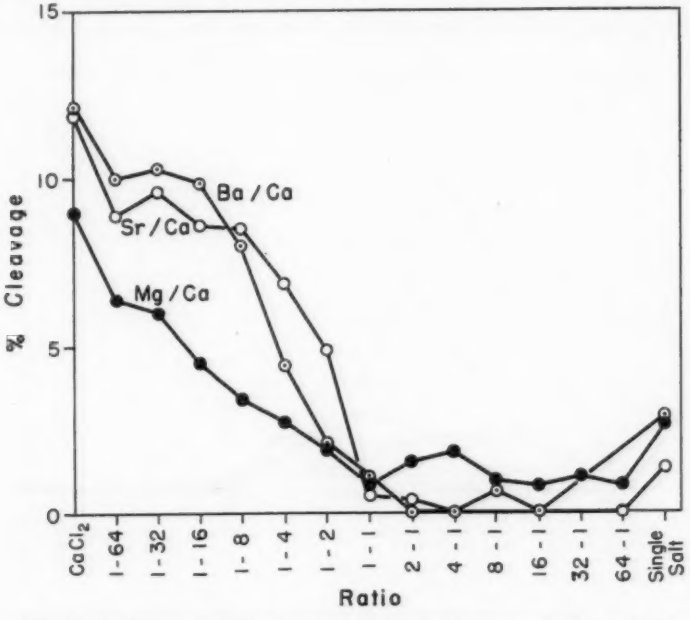

FIG. 4. Action on *Arbacia* eggs of isotonic solutions of some bivalent cations singly and in various mixtures and proportions.
Mg/Ca—average of eight experiments.
Sr/Ca—average of eight experiments.
Ba/Ca—average of eleven experiments.

there has been no cytoplasmic division. There is a marked increase in the percentage of activation as the proportion of sodium increases up to about ratio 1–4 followed by a marked decrease, with no activation in mixtures in which the ratio is above 16–1 or in the Na solution. Isotonic LiCl solution is able to activate a small percentage of *Arbacia* eggs. The degree of activation increases rapidly as the proportion of lithium increases beginning in a ratio of about 1–16, with the highest percentage at a ratio of about 1–2, followed by a sharp decline at a ratio of about

2–1. Isotonic $MgCl_2$ is able to activate a very small percentage of eggs. There is a very great increase in the percentage of activation as the proportion of sodium increases up to an average ratio of 1–8 followed by an equally sudden decrease with activation ceasing at a ratio of about 8–1. This result is of interest because there is a marked increase in the percentage of activation both in Na/Ca mixtures and Na/Mg mixtures between ratios of about 1–32 and 1–8.

Effect of Mixtures of Calcium Chloride and Some Bivalent Cations. —The result of experiments in which *Arbacia* eggs are exposed to solutions of magnesium, strontium or barium chloride and calcium chloride in various proportions is shown in Fig. 4. While magnesium has a slight activating effect, there is a mutual anatagonism between calcium and magnesium. Strontium is able to activate a small percentage of eggs but is much less effective than calcium. The degree of activation by calcium decreases gradually as the proportion of strontium increases up to a ratio of about 1–8 with a sharp decrease above a ratio of 1–8. It may be concluded that there is an antagonism of calcium by strontium. Barium alone is able to activate a small percentage of eggs. The degree of activation decreases as the proportion of barium increases with activation ceasing at a ratio of about 2–1. It may therefore be concluded that there is antagonism of calcium by barium. There are many eggs with nuclear divisions without cytoplasmic cleavage in mixtures where the Ba/Ca ratio is between 32–1 and 64–1.

In summary, Na and K are not effective in activating *Arbacia* eggs while Li activates a small percentage. The monovalent cations increase the percentage of activation by calcium when present in certain definite proportions. The bivalent cations, Sr, Ba and Mg each have an inhibiting effect on activation by Ca and the antagonism is mutual. There is even more variation in the behavior of *Arbacia* eggs than in *Cumingia* eggs but again it is a variation in magnitude rather than in kind (see original paper).

Discussion

The problem of the activation of unfertilized eggs by an alteration of their chemical environment has been vigorously attacked by a number of investigators. R. S. Lillie (1910, 1911*a*, 1911*b*) was the first to report activation of marine eggs by means of isotonic salt solutions. To date, the activation by isotonic salt solutions of the eggs of seven species of marine invertebrates has been reported. In each case, activation was accomplished by exposing eggs to varying combinations and proportions of the chlorides of the cations of sea water. In all, the presence of Ca appears to be essential while there is variation in the element which it is necessary to add.

R. S. Lillie (1910) reported the initiation of development in *Arbacia* eggs when exposed to isotonic NaCl for varying periods of time followed by return to sea water. He reported no activation of *Arbacia* eggs when exposed to isotonic solutions of $CaCl_2$, $SrCl_2$ and $MgCl_2$ followed by return to sea water. In the present work the opposite of these observations is reported. The difference in results obtained with the monovalent cations may be due to the fact that in the present work the eggs were not returned to sea water and the difference in results obtained with bivalent cations may be due to the fact that *Arbacia* eggs must be exposed to isotonic solutions of $CaCl_2$, $SrCl_2$ and $MgCl_2$ for hours in order to obtain a noteworthy percentage of activation.

The results of the experiments on the eggs of *Arbacia* reported in this paper are in agreement with the results of the work on marine eggs reported by Dalcq (1928) on *Barnea candida,* Hobson (1928) on *Thalassema neptuni* and Pasteels (1935) on *Phascolion* and *Hydroides* where alkalinity and the monovalent cations K, Na and Li increase markedly the percentage of activation of *Arbacia* eggs by Ca and are necessary in order to obtain a high percentage of activation of the eggs of most individuals and where cleavage appears to be more nearly normal in favorable binary mixtures than in isotonic $CaCl_2$ alone.

However, the results on activation of the eggs of *Cumingia* by isotonic salt solutions are not in agreement with the results of the work reported by Dalcq, Hobson and Pasteels on activation of marine eggs by isotonic salt solutions. The addition to the calcium solution of excess OH ions or of the monovalent cations Na or K does not increase the percentage of activation of *Cumingia* eggs but has the opposite effect. The segmenting eggs appear more nearly normal and more healthy in isotonic $CaCl_2$ alone than in any of the binary mixtures used. Activation by isotonic $CaCl_2$ with respect to time of polar body formation and percentage of activation compares favorably with activation of eggs by sperm. We may say that in the case of *Cumingia* eggs, Ca is the sole activating agent and that no other external agent or treatment is necessary.

There are several theories to explain the activation of unfertilized eggs. All these theories are aspects of more general theories of stimulation. The oxidation theory of activation was stated by J. Loeb (1913). It is now quite certain that not all activating agents increase the rate of oxidation. Heilbrunn (1915) pointed out that cyanide does not prevent the first stages of development in *Arbacia* eggs and (1920*a*) that maturation in *Cumingia* eggs is not dependent on an increase in oxygen consumption. Whitaker (1931, 1932) reported that in the eggs of *Nereis* and *Arbacia* there is an increase in the rate of respiration following fertilization whereas in the eggs of *Chaetopterus* and *Cumingia* there is

a decrease following fertilization. Activation of eggs by an isotonic solution of $CaCl_2$ can scarcely be due to an increase in the rate of oxidation for calcium is usually thought to decrease the rate of oxidation (see for example, Ahlgren, 1925; Holck, 1934; and Thunberg, 1937). It may therefore be concluded that rate of oxygen consumption is not the primary factor in the initiation of development of eggs.

The permeability theory of activation, founded by R. S. Lillie (1916, 1917, 1918) has been used to explain initiation of development in eggs. That there is an increase in permeability in some marine eggs following activation has been convincingly demonstrated by a number of careful investigators. The work of Lillie (1916, 1917, 1918) and McCutcheon and Lucké (1932) shows that the permeability of *Arbacia* eggs to water increases after fertilization and the work of Stewart and Jacobs (1932) shows that permeability of these eggs to ethylene glycol increases after fertilization. However, activation of eggs by isotonic $CaCl_2$ cannot be conceived of as due to an increase in permeability. It is universally agreed among students of permeability that bivalent cations such as magnesium and calcium cause a decrease in cellular permeability and antagonize those reagents known to increase it. Therefore the action of calcium in the activation of *Cumingia* eggs cannot be explained on the basis of the permeability theory.

Dalcq (1924*a*, 1924*b*) has developed a depolarization theory of activation. This theory depends upon the presence of charges of definite sign upon the cortex and constituents of the egg and upon the existence of a potential gradient on the cortex. He concluded that a disturbance of the intraovular cations results in depolarization, that Ca is the most effective agent in bringing about depolarization and that activation is the result of depolarization. However, the depolarization theory of Dalcq seems highly speculative and is difficult to understand from the electrochemical standpoint.

Heilbrunn (1915) favored the coagulation theory of activation. This theory, which is now termed the colloid chemical theory is, as the permeability theory, a broad theory of stimulation for all types of irritable systems. In a study of the chemical changes in the egg following activation, Heilbrunn and his students have shown that whenever a cell is stimulated, Ca is released from the cortex. Heilbrunn and his students have further shown that if Ca is first removed from egg cells by oxalate, stimulating agents are not effective but that upon the return to sea water the usual response may be obtained (see Heilbrunn and R. A. Young, 1930; Heilbrunn and K. Wilbur, 1937). For a full discussion of the colloid chemical theory of stimulation see Heilbrunn's "Outline of General Physiology," 1937. The results of the study of the activa-

tion of the eggs of *Arbacia* by favorable binary mixtures of bivalent and monovalent cations and of the study of the activation of the eggs of *Cumingia* by isotonic solutions of bivalent cations alone, where 100 per cent of the eggs of some individuals undergo apparently normal cleavage in a period of time which compares favorably with the time of activation of eggs activated by sperm, favor the colloid chemical theory of Heilbrunn and are directly opposed to any interpretation in terms of the oxidation or permeability theories.

Summary

1. When unfertilized *Cumingia* eggs are placed in 0.3 M $CaCl_2$, 100 per cent of the eggs of some individuals undergo apparently normal cleavage. The time of polar body formation and of the first cleavage in eggs activated by Ca is approximately the same as the time of polar body formation and of cleavage in eggs activated by sperm.

2. Polar body formation and cleavage in *Cumingia* eggs proceed normally in 0.3 M $CaCl_2$ at the various pHs between pH 6.2 and pH 8.6 but are inhibited above and below this range.

3. The bivalent cations Sr, Ca and Ba are able to activate *Cumingia* eggs and are named in the order of their effectiveness.

4. The time of polar body formation in *Cumingia* eggs activated by isotonic solutions of $SrCl_2$ and $BaCl_2$ is approximately the same as the time of polar body formation in eggs activated by sperm.

5. The monovalent cations K and Na and sea water inhibit activation of *Cumingia* eggs by Ca. The percentage of activation decreases as the K/Ca, Na/Ca and sw/Ca ratios increase. K has a greater inhibiting effect than Na.

6. Ba inhibits the activation of *Cumingia* eggs by Ca, Sr inhibits very slightly the activating effect of Ca, while Mg does not appear to have an inhibiting effect on activation of eggs by Ca.

7. When unfertilized *Arbacia* eggs are placed in 0.3 M $CaCl_2$, from 40 to 60 per cent of the eggs of most individuals undergo cleavage if the pH of the solution is between 8.8 and 9.2. No membrane is elevated in isotonic salt solutions.

8. Below pH 8.8 the percentage of cleavage is low and above pH 9.0 the percentage of cytolysis is high in *Arbacia* eggs activated by isotonic $CaCl_2$.

9. Isotonic solutions of $SrCl_2$, $BaCl_2$ and $MgCl_2$ are able to activate a certain percentage of *Arbacia* eggs, but these ions are not so effective as Ca and their action is somewhat variable.

10. The monovalent cations Na, Li and K in certain definite proportions increase the percentage of cleavage induced by Ca while in other

proportions they have the opposite effect. Similarly isotonic NaCl, in certain definite proportions increases markedly the percentage of activation by the Mg solution while in other proportions Na has the opposite effect.

11. Sr, Mg and Ba inhibit the activation of *Arbacia* eggs by Ca, the inhibiting effect increasing as the Sr/Ca, Mg/Ca and Ba/Ca ratios increase.

12. The results are brought into relation to the colloid chemical theory of stimulation.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1941

JULY 8

The source of pancreatic juice bicarbonate. Eric G. Ball.

(This paper has already appeared in full in the July, 1941 number of the *Journal of Biological Chemistry.*)

The permeability and the lipid content of the erythrocytes in experimental anemia. Arthur J. Dziemian.

In a series of albino rabbits the permeability of the erythrocytes to glycerol, diethylene glycol, ammonium propionate and ammonium salicylate was studied and the total lipid, cholesterol and phospholipid contents of the red cells, calculated per ml. of cells, per erythrocyte and per square micron of cell surface, were determined. The rabbits received subcutaneous injections of 50 mg. of phenylhydrazine hydrochloride and changes in the permeability of the red cells to the above substances were studied. Within about nine days after injection, practically an entirely new population of red cells was present in the animals, as shown by a study of changes in cell diameters. At this time the rate of penetration into the cells of diethylene glycol and glycerol had greatly increased, while the ammonium propionate and salicylate penetrated slower than normal. Thereafter the times of 50 per cent hemolysis of the red cells in all the solutions used returned slowly toward the original values. When lipid analyses on the red cells of the experimental animals were made, no correlation was found between the changes in permeability and the changes in the lipid content of the erythrocytes.

The rectifying property of the giant axon of the squid. Rita Guttman and Kenneth S. Cole.

Measurements of the resistance of the squid giant axon have been made by means of a direct current Wheatstone bridge between one injured end of the axon, placed in KCl, and the other end immersed in sea water, with the inter-electrode region hanging in oil. During the passage of small currents through the axon, the resistance of the fiber does not depend upon the magnitude and direction of the current. But when larger currents are used this is no longer true, i.e. if the uninjured end is the anode, the over-all resistance of the axon is greater than that found during the passage of small currents while at the cathode the reverse is true. The apparent resistance of the nerve fiber, then, depends upon the magnitude and direction of the current flowing through it. The nerve fiber thus does not obey Ohm's Law and is an electrical rectifier which permits current to pass more easily in one direction than the other, rather than a pure resistance.

Cocaine and veratrine cause progressive and reversible loss of rectification. As the axon dies, excitability is lost, and the rectification and the resting potential disappear. When the fiber is completely dead, there is no rectification and the fiber acts as a pure resistance.

Such a rectification is to be expected if the membrane conductance is a measure of ion permeability and this permeability is increased at a cathode and decreased at an anode. Also, rectification has been suggested as an explanation of some electrotonic and excitation phenomena.

Metabolism and fertilization in the starfish egg. Herbert Shapiro.

The respiration of immature, mature and fertilized eggs of the starfish, *Asterias forbesii*, was studied during May and June by means of Warburg manometers, over a temperature range 11.5 to 27.8° C. Oxygen uptake by unfertilized eggs may remain constant for periods as long as ten hours. Fertilized eggs show a relatively constant rate at first, and then a slowly increasing one, as embryological development advances. An average increase of approximately 30 to 50 per cent was found in the rate of fertilized eggs, as compared with unfertilized, the rise being slightly higher at the low temperatures.

Although some experiments yielded results in agreement with those of Loeb and Wasteneys (*Arch. entw.-mech. Organism.*, **35**: 555, 1912) and of Tang (*Biol. Bull.*, **61**: 468, 1931) in showing little or no change after fertilization, the more extended series reported here, done on eggs showing high percentages of cleavage, and during the optimal portion of the breeding season, demonstrate that the average starfish egg undergoes a significant acceleration of oxidations subsequent to fertilization.

JULY 15

Factors in the lunar cycle which may control reproduction in the Atlantic palolo. L. B. Clark.

Of all the physiological influences attributed to the lunar cycle, the coincidence of reproduction of certain marine polychaetes with specific phases of the moon has been best determined. Of such animals, the palolo worms are perhaps outstanding because of their size, striking reproductive behavior, and the apparent specific relation between the moon's phases and time of reproduction.

A number of experiments on artificially changing the light relations of the lunar cycle by illuminating or shading rocks containing worms were undertaken. The results of all the experiments are consistent in that if the average duration of light is increased, reproduction occurs before the controls, and if the average duration of moonlight is decreased, the time of swarming occurs after the controls or not at all. It is concluded, therefore, that this is a factor involved in reproduction and that the effectiveness of the various phases of the moon's cycle is correlated with the average duration of moonlight during the cycle.

If this were the only factor involved, the effectiveness of moonlight to induce swarming would increase to a maximum about three days after the full moon and then decrease. But the effectiveness of moonlight is bimodal, the modes centering about the first and last quarter moon, with the latter much more effective. Obviously there must be some other factor operating in moonlight. The only other factor varying in the desired manner is the daily difference in the rate of change of moonlight. This reaches a maximum at the new and full moons and a minimum at the first and third quarter. If it is postulated that the effectiveness of moonlight in determining the time of swarming bears some correlation to the reciprocal of the difference in the daily rate of change of moonlight, the resultant varies in a manner similar to the incidence of swarming during the lunar cycle.

Accumulation of manganese and the sexual cycle in Ostrea virginica. Paul S. Galtsoff.

Oysters of known age and origin, planted on an experimental bottom in Long Island Sound near Milford, Connecticut, showed a distinct annual cycle in the Mn-content which varied from 7.3 to 51.0 mg. per kilo d.w. During the twenty-nine months' period of observations the high Mn content (from 30 to 50 mg. p.k.d.w.) invariably coincided with the period of gonad development and sexual activity of the oysters (May–August) while low Mn content (from 7 to 11 mg.

p.k.d.w.) occurred in winter and early spring (November–April). Ovaries were found to be particularly rich in Mn (51.0–59.6 mg. p.k.d.w.) and testes contained only from 4.6 to 7.2 mg. p.k.d.w. Other tissues contained the following amounts: gills, 17 to 18 mg. p.k. in winter and from 35 to 38.6 in summer; mantle, 8.7 in January and from 14.2 to 17.0 in September; visceral mass, 8.9 to 18.4; and adductor muscle, 4.3 to 5.2 in July and 4.1 to 9.3 in January. These results indicate that the Mn cycle is associated with the development of a female phase of the oyster. The physiological role of the metal in the metabolism and its relation to the sex change in this species is not known.

Studies on the life history of Siphodera vinaledwardsii, a trematode parasite of the toadfish. R. M. Cable and A. V. Hunninen.

Experimental studies on the life history of *Siphodera vinaledwardsii* (Linton) have demonstrated that this trematode is related to the Heterophyidae as postulated by Manter, Price and Wilhelmi on the basis of morphological and serological investigations. The definitive host in the Woods Hole region is the toadfish, *Opsanus tau*, practically all of which are naturally infected. The small marine snail, *Bittium alternatum*, serves as the molluscan host in which the cercariae develop in simple, elongate radiae. The cercaria is a pleurolophocercous form of an unusual type since the tail is inserted ventrally and coiled when at rest, the fourteen penetration glands have two instead of the usual four bundles of ducts in the region of the oral sucker, and the excretory formula is $2[(2+2)+(2+2)]=16$ flame cells. The cercariae penetrate and encyst in various species of flounders, developing into apparently infective metacercariae in approximately two weeks. Cysts occur in the fins, body wall and even the myocardium of the flounder. Feeding experiments thus far completed indicate that the toadfish becomes infected by eating fish containing metacercariae. Three toadfish, isolated for four weeks, were fed fish containing 13-day metacercariae. Two of these have been examined to date and found to harbor large numbers of very young worms in addition to a few mature specimens from previous natural infection.

Pathology and immunity to infection with heterophyid trematodes. Horace W. Stunkard and Charles H. Willey.

The term heterophyid refers to a large family of digenetic trematodes which infect fish-eating birds and mammals. *Cryptocotyle lingua* is a common heterophyid species in the Woods Hole area; its life cycle was reported by Stunkard (1930). The larval stages are produced in *Littorina littorea* and *L. rudis*, while the cercariae encyst in the cunner and other fishes.

Stunkard and Willey (1929) studied the development of *C. lingua* in cats and rats. In these hosts, the worms developed to sexual maturity between the intestinal villi and no intramucosal invasion was observed. Since there is evidence to indicate that cats and rats are not favorable hosts, the studies were continued on terns and dogs.

Young terns developed a very severe infection from the sixth to the fourteenth day, when the number of eggs in the feces began to diminish. After the twentieth day the feces contained very few eggs and large numbers of young worms recently liberated from their cysts. After an initial heavy infection, gulls and terns develop a strong resistance to superinfection and the presence of a few worms serves to maintain a substantial immunity.

A dog, fed enormous numbers of cysts, began to pass eggs of the parasite on the fifth day. Large numbers of immature and mature worms were present on the surface of the mucosa and in the crypts between the villous folds. The villi showed acute inflammatory changes, desquamation, hyperemia, and excessive mucous secretion. There was no invasion of the intestinal glands or tunica propria. An-

other dog, similarly fed for fourteen days, was in a moribund condition and autopsy revealed the presence of thousands of sexually mature worms. Dogs were given moderate infections and allowed to recover. Eggs began to appear in the feces on the fifth day, were numerous for about four weeks, after which the number began to decline. At the end of three months very few eggs could be found and the feces were negative at the end of six months. After resistance had been established in dogs, the feeding of large numbers of metacercariae produced no visible ill effects and very few eggs appeared in the feces.

These experiments show that birds and dogs, if the latter survive an initial infection, effect a "self-cure" (as that term was defined by Stoll, 1929) and thereafter are resistant to any substantial reinfection.

JULY 22

The organization of the melanophore system in bony fishes. G. H. Parker.

Catfish color changes, which range from pale yellowish-green to coal-black, are mediated in the main by three receptors, the dorsal retina, the ventral retina, and the skin. The pale phase of this fish is excited through the dorsal retina, which is best illuminated when the fish is on a white background lighted from above. Chromatic nerve tracts extend from the dorsal retina through the central nervous organs and the autonomic system to the melanophores. The final fibers in these tracts are adrenergic in that they discharge adrenaline which blanches the fish by causing melanophore pigment concentration. The fiber tracts concerned with this response may be designated as the retino-adrenergic arc. The blood of a pale catfish is devoid of the darkening agent intermedine, a state which indicated the inhibition of the intermediate pituitary lobe. From the dorsal retina nerve tracts presumably extend through the central nervous organs to the pituitary gland and thus mediate pituitary inhibition. Such tracts may be called the retino-pituitary inhibition arc.

The dark phase of the catfish is induced through the ventral retina and the skin. When the fish is on a black background the ventral retina is excited by light from above. From it impulses pass through the central nervous organs and the autonomic system over whose cholinergic fibers they reach the melanophores. Here the resultant acetylcholine excites the melanophores to disperse their pigment and thus to darken the fish. The tracts concerned with these activities constitute the retino-cholinergic arc.

The second receptor concerned with the dark phase of the catfish is the skin, which can be studied best in blinded fishes. Such fishes, dark, intermediate, or pale, if put at once into darkness retain their original tint for days, but on being exposed to daylight quickly become coal-black. From the photoreceptors in the skin nerve-fibers pass in tracts to the pituitary gland which is thereby excited to discharge intermedine. This then passes by way of the blood to the melanophores whose dispersed pigment darkens the fish. These nerve tracts and blood courses may be called the dermo-pituitary arc. Other bony fishes whose melanophore systems are much like that in the catfish are the angelfish, eel, snakefish, Japanese catfish and the stickleback. The killifish and the flatfishes are similar but lack effective pituitary organs.

Some aspects of pigment deposition in feather germs of chick embryos. Ray L. Watterson.

A study of the developmental history of melanophores in the wing skin and feather germs of Barred Rock embryos indicates that pigment deposition is not a function of pigment cells alone, but is definitely controlled by recipient barbule cells. (1) Melanophores are packed with pigment granules early in development. Nevertheless, pigment is not distributed to epidermal cells until certain of them

become visibly differentiated as barbule cells. (2) Pigment granules accumulate at the tips of pigment cell processes and become pinched off, whereupon they lie freely among the epidermal cells. Pigment liberated in this manner is later taken up by barbule cells. (3) Pigment is deposited in each row of barbule cells in a definite sequence. The most peripheral barbule cells are the first to elongate and to form keratin; only when these visible differentiation processes begin can they receive pigment. As this wave of differentiation spreads toward the pulp, more axial cells become capable of receiving pigment. (4) A study of the development of down feathers with split barb-vanes indicates that pigment cell processes are specifically attracted toward barbule cells. In their development barbule cells differentiate in the center of a barb-vane ridge where they normally do not occur. Melanophore processes leave their normal paths, extend toward these centrally located cells and carry pigment to them. (5) Pigment deposition stimulates the melanophores involved to undergo proliferation. Melanophores undergoing mitotic division occur almost exclusively at those levels of feather germs where pigment deposition is in progress. (6) Feather germs elongate slowly until 10 days and 18 hours, whereupon they elongate rapidly, attaining their full growth by 13 days. The onset of pigment deposition coincides with the onset of rapid growth. Lillie and Juhn have estimated that 90 per cent of the axial growth of regenerating feathers is accomplished by cell elongation. Pigment deposition begins at that phase of development when barbule cells begin to elongate rapidly.

The influence of hormones on the differentiation of melanophores in birds. Howard L. Hamilton.

When explants of skin from embryos of birds which have red and black pigments in their feathers are grown in a tissue culture medium consisting of blood plasma and embryonic extract, black melanophores appear but red ones occur very infrequently. If sex hormones are added to the culture medium, then many red melanophores as well as black ones differentiate in the explant. The two kinds of pigment cells are recognized as discrete cell types because of color, shape, and solubility differences in the granules, viscosity differences in the cytoplasm as shown by more rapid granule movement in red melanophores, and differences in their reactions to various hormones. In general, sex hormones increase the number of red melanophores which differentiate in treated explants from red breeds. Sesame and olive oils also produce a stimulation (possibly due to traces of sterols). Black melanophores are inhibited by estradiol, but estrone and testosterone favor their differentiation.

Desoxycorticosterone, an adrenal cortical hormone, decreases the number of melanophores in the New Hampshire Red, White Leghorn, and Barred Rock breeds of fowl. Sex hormones produce a similar inhibition of black melanophores in the latter breed. Young tissue (5–6 days) yields few or no melanophores when grown in the presence of hormone; in older tissue (7–8 days) there is a reduction in number of melanophores and inhibition of feather germ formation as well. Crystalline hormones act apparently by slowing the process of melanin synthesis, so that the treated cells, although chronologically of the same age as the controls, are physiologically younger. It is concluded that genetic differences in the precursor cells must determine whether they become red or black melanophores, but that environmental factors (physiological differences in feather germs; hormones) may directly influence which of the two kinds will predominate.

The distribution and development of the melanophore hormone in the pituitary of the chick. Hermann Rahn.

In the chicken a structural pars intermedia is absent. The melanophore hormone, however, is present in considerable quantities and is found in greatest concen-

tration in the region of the pars anterior furthest removed from the infundibular process (Kleinholz and Rahn). Quantitative assays of the melanophore hormone were made on the pituitary of chicks throughout the whole development. The Anolis lizard test was used for these determinations. The first appearance of the hormone can be detected on the fifth day of incubation, i.e. five days before differentiation in the pituitary can be seen by ordinary cytological methods. During the last half of the incubation period the melanophore hormone per unit weight of pituitary tissue increases rapidly and reaches its greatest concentration at hatching time. All further apparent increase in hormone per total gland after hatching time can be accounted for by the increasing growth or weight of the pituitary gland.

JULY 29

Effect of sea water on the radiosensitivity of Arbacia sperm. T. C. Evans and J. C. Slaughter.

The percentage fertilization resulting from inseminations with sperm irradiated "dry" is much greater than that irradiated in sea water. The amount of injury to the sperm increases with dilution. For example, to reduce the fertilizations to 50 per cent, sperm diluted to 1:100 must receive 20,000 roentgens, whereas a suspension of 1:1000 needs only 3,000 r.

The sperm are more susceptible immediately after the addition of sea water, when the rate of oxygen consumption is high, than thirty minutes later when the rate of oxygen consumption is lower.

Concentrations of as low as 0.01 per cent egg albumen greatly increase the radioresistance of sperm in sea water. The resistance in sea water is also increased upon the addition of sufficient amounts of dead *Arbacia* sperm or living sperm of *Nereis*.

It therefore appears that the condition of the medium during irradiation markedly affects the radiosensitivity of the sperm as measured by the decrease in percentage fertilization.

The fractionation of cellular respiration by the use of narcotics. Kenneth C. Fisher.

An examination has been made of new data and of data from the literature concerning the effects of narcotics on oxygen consumption. The relation between concentration and effect seen in all these cases possesses a feature which suggests that the inhibitor affects independently two discrete parallel respiratory systems in each of the preparations. In general the concentration range over which the effects are graded is not identical for the two systems.

Inhibition of cell division in yeast, a protozoön, the sea-urchin egg and of light production in luminous bacteria, approaches completion at narcotic concentrations which affect oxygen consumption in the same preparation relatively much less. Actually, inhibition of these activities parallels closely inhibition of the more sensitive of the two respiratory systems.

One substance, benzoate, has been found for which the sensitivities of the two respiratory systems in yeast are just the reverse of those shown for narcotics, i.e., as the concentration of benzoate is gradually increased, the "second" system is inhibited before the "first." As would be expected ideally, this compound inhibits oxygen consumption approximately 55 per cent before beginning to affect the rate of cell division.

It is concluded that in many cells the oxygen consumption, at least at the site of action of narcotics and allied substances, is composed of two independent parallel respiratory systems. The metabolism for cell division and light production appears to be associated with only one of these two.

These observations have been made with the collaboration of Messrs. J. R. Stern, R. J. Henry and Richard Ormsbee.

Effect of azide on Cypridina luciferin. Aurin M. Chase.

There are a number of pieces of information now available which bear upon the chemical nature of *Cypridina luciferin.* Anderson (1936) has suggested a resemblance to certain naturally occurring hydroxy-benzene derivatives studied by Ball and Chen (1933), on the basis of the similarity in oxidation-reduction potential. Chase (1940) has described changes in the visible absorption spectrum during oxidation of luciferin which indicate a possible quinoid structure. Giese and Chase (1940) have postulated an aldehyde or keto group in the luciferin molecule on the basis of an irreversible combination with cyanide. Chakravorty and Ballentine (1941) have proposed as a partial structure for the luciferin molecule a hydroquinone nucleus with a keto-hydroxy side chain. The hydroquinone nucleus would explain the reversible, non-luminescent oxidation of luciferin and the keto group would be the point of combination with cyanide or with luciferase.

Giese and Fisher (unpublished data) have described inhibition of luminescence in luminous bacteria by sodium azide and this observation prompted the present study of the effects of azide on luminescence of purified *Cypridina luciferin* and luciferase. At pH 6.6 luminescence is found to be reversibly inhibited at azide concentrations from about 0.005 to about 0.1 M. At pH 5.4 these same azide concentrations are much more effective, indicating that the HN_3 may be the active agent. The effect appears to be chiefly upon the luciferin. Plotted in terms of the mass law equation, the data fall upon straight lines with slopes approximately equal to *one.*

It is tentatively suggested that HN_3 reacts with luciferin in the same way that it has been shown by Fieser and Hartwell (1935) to react with benzo- and naphthaquinones. Further evidence for a quinoid group in the luciferin molecule would therefore be indicated.

August 5

Aging phenomena, and factors influencing the longevity of Mactra eggs. Victor Schechter.

(This paper has appeared in the *Jour. Exper. Zoöl.,* Vol. 86, No. 3, for 1941.)

Comparison of the respiratory rates of different regions of the chick blastoderm during early stages of development. Frederick S. Philips.

As an introductory study of the chemical processes involved in the regional differentiation of the chick blastoderm (the area pellucida of the head-process embryo), the rates of oxygen consumption were studied of various isolated pieces. In addition, the respiratory rate of pieces containing most of the pellucid area was determined at various stages of development from the unincubated blastoderm to the 12-somite embryo. Oxygen consumption was measured in the Cartesian diver microrespirometer. The total-nitrogen of the tissues was estimated by a modification of the Conway micro-Kjeldahl procedure.

The pellucid area of head-process embryos was divided into pieces containing respectively the head-process, node and anterior streak, middle streak, posterior streak, and right and left lateral regions. The Qo_2' (m.μl. O_2 consumed/hour/γN) of all these regions is essentially similar. No major differences are apparent in the rate of oxygen consumption of the various regions studied which can be correlated with their marked regional differences in developmental potency.

The Qo_2' of the embryonic area increases from a value of about 33 in the unincubated blastoderm until it reaches a value of about 75 in the 17-hour embryo, the early definitive streak stage. The head-process, 5-6-somite, and 11-12-somite embryos have rates of oxygen consumption similar to that of the 17-hour embryo.

During this period of development from the unincubated to the head-process stage the total quantity of nitrogenous material in the whole area pellucida increases only slightly. However, the total amount of O_2 consumed/hour increases at least threefold. Apparently, therefore, the increase in respiratory rate during the first 17 hours of development depends on the large increase in cell-number coincident with the conversion of intracellular yolk material into active cellular constituents.

Further interpretations of the effects of CO and CN on oxidations in living cells. Mathilda M. Brooks.

In these experiments, the rate of O_2 consumption, of cleavage and length of life of eight different stages in the development of sea-urchin and starfish eggs, as affected by certain accelerators and inhibitors, was studied. The stages included unfertilized eggs, first cleavages, morula, blastula, early gastrula, late gastrula, early pluteus and late pluteus. The reagents were methylene blue (.00012 M); KCN (.00025 M) and CO as near 100 per cent as possible. It was found that methylene blue accelerated O_2 consumption in the early stages, decreased it or had no effect in the middle stages and increased it again in the late stages. The decrease produced by KCN varied, so that the rate varied from 63 per cent of the normal to about 6 per cent depending upon the stage of development; the decrease produced by CO varied causing a rate of 92 per cent of the normal in certain stages and a rate as low as 20 per cent in others. Methylene blue accelerated the rate produced by CO about 10 per cent, and either increased it when KCN was used or produced no effect.

In the case of cleavage, KCN produced multiple aster formation without cell division even in the presence of methylene blue at this concentration. Methylene blue prevented cytolysis produced by CO; doubled the life of the embryo; caused a faster rate of development; and increased the size of the pluteus stage of *Arbacia* from 280 μ (controls) to 420 μ.

These experiments suggest new aspects of the respiratory enzymes and associated redox systems. Since methylene blue poises the potential, it appears that the optimum redox potential at which these systems function changes with development. This can be interpreted either that the respiratory enzyme assumes a different rôle or that these enzyme systems are actually different at the various stages of development.

August 12

Studies on conditions affecting the survival in vitro of a malarial parasite (Plasmodium lophurae). William Trager.

The malarial protozoa are obligate intracellular parasites which have never been cultured *in vitro*. Indeed, little has thus far been discovered concerning even the most elementary conditions which might favor their survival outside of their living host. Accordingly, a series of experiments was conducted in which parasitized blood cells taken from a chicken infected with *P. lophurae* were placed in various media and their time of survival at 40–42° C. determined. The chief criterion of survival was the ability of the parasites to infect baby chicks under a set of standard conditions. It was found that survival *in vitro* was favored by aeration but not by a very high oxygen tension, by a balanced salt solution of high potassium content, by certain concentrations of glucose or glycogen, by glutathione, by red cell extract, by low concentrations of chick embryo extract and chicken liver extract, by daily renewal of the medium and by an optimal density of parasites per cu. mm. In the best preparations, as judged by infectivity, at least 40 per cent of the original parasites were alive on the third day, at least 20 per cent on the fourth day, about 1 per cent on the fifth day and about 0.05 per cent on the sixth day. In these preparations there was a small increase in parasite number during the first day of incubation.

The effect of dyes on the response to light in Peranema trichophorum. Charles C. Hassett.

When stimulated by a sudden increase in the intensity of light, the flagellate *Peranema trichophorum* responds by a shock reaction, i.e., it ceases forward motion and bends sharply, then moves off at an angle to its original direction of movement. The time required to produce this response was used as a measure of the photodynamic effect of a number of dyes. The average reaction-time of untreated peranemae was found to be 12.1 seconds; the optimum concentration of active dyes (ca. 5×10^{-4} M), decreased this to ca. 1.0 second; weaker solutions of these dyes and all solutions of less active dyes produced longer reaction-times, with 12 seconds as the approximate maximum. The order of effectiveness of the dyes was: rose bengal, eosin, neutral red, methylene blue, Nile blue sulfate, auramine O. Orange G had no effect. Brilliant green increased the reaction-time to 16.2 seconds in 5×10^{-5} M solution; weaker solutions produced shorter reaction-times down to 12.4 seconds at 1×10^{-7} M. This may have been due to the greater toxicity of brilliant green.

These results indicate that (1) the photodynamic action of dyes can affect the response of *Peranema* to light; (2) the fluorescence of a dye is not a measure of its photodynamic effect; (3) there is no correlation between the wave-length of the light absorbed by a dye and its effect on the response of *Peranema;* (4) dyes with very different molecular structures produce similar effects.

The utilization of ammonia by Chilomonas paramecium. John O. Hutchens.

Using a solution containing CH_3COONa, NH_4Cl, $(NH_4)_2SO_4$, K_2HPO_4, $CaCl_2$, $MgCl_2$, $FeCl_3$, and thiamin hydrochloride in which *Chilomonas* attains populations up to 10^6 cells/cc., sufficient quantities of cells have been obtained to permit accurate analyses of the composition of the cells. Also growth is accompanied by sufficient conversion of substrates to permit the compilation of balance sheets.

All data deal with cells and the conversions achieved by them during the logarithmic phase of the growth curve. The results of the experiments are as follows: 10^6 cells weigh 2.5 mg. wet and 0.61 mg. dry, i.e. they are 24 per cent solid material; 2.5 per cent of the wet weight is tungstic acid precipitable nitrogen, therefore the protein ($N \times 6.25$) accounts for 16 per cent of the wet weight or 67 per cent of the solids. Of this nitrogen 28 per cent is amide nitrogen, indicating a high percentage of dicarboxylic amino acids in the protein. Ammonia utilized by the chilomonads (up to 27 per cent of the original ammonia in the solution) is all recoverable by Kjeldahl digestion. No significant amounts of ammonia are oxidized to nitrate or nitrite. Therefore, while ammonia is a satisfactory source of nitrogen for *Chilomonas,* it is not utilized as a source of energy in the solution containing acetate.

The possibility of thiamin synthesis by ciliates. Virginia C. Dewey and G. W. Kidder.

The only ciliate so far investigated, *Tetrahymena geleii,* has been reported to require the whole thiamin molecule for growth. Using a vitamin-free casein basic medium, in which no growth of the ciliate occurred, it was found that the addition of thiamin permitted extremely slow, but transplantable growth. The addition of alfalfa or timothy hay extract to the casein permits optimum growth. In an attempt to make a quantitative estimate of the thiamin requirement, the alfalfa supplement was treated to destroy the thiamin by heating at 121° C. at pH 10 or higher for periods of 1–3 hours. The treated supplement, when added to casein, permitted optimum growth of *T. geleii* even in the absence of thiamin. This is

true also of *T. vorax* with regard to alfalfa, but not timothy hay. *Glaucoma scintillans,* however, requires both thiamin and the alfalfa supplement. The same experiment was repeated using proteose-peptone treated to destroy the thiamin as a basic medium with essentially the same results. Four possible explanations of this phenomenon are offered: (1) heat treatment does not destroy thiamin, which seems unlikely, since thiamin must be added to the medium besides the alfalfa in order to obtain growth of *Glaucoma;* (2) there is some substance in plant material which, acting catalytically, permits the resynthesis of thiamin from the fragments formed by the heat treatment; this seems most likely at present; (3) there are present in plant materials thiazole-like substances more resistant to heat than the thiazole of thiamin and which can take the place of the latter in the thiamin molecule; there is no evidence for or against this possibility; (4) there is some entirely different substance in plant materials which can substitute for thiamin. This last possibility seems to be excluded by the fact that *Glaucoma* will grow in the casein-heat treated alfalfa medium after it has supported a population of *Tetrahymena* which were killed by heat before inoculating the *Glaucoma.* This indicates synthesis of thiamin by *Tetrahymena.*

August 19

Electrical potential and activity of choline esterase in nerves. David Nachmansohn.

(Some of this material is scheduled to appear in the September, 1941 issue of the *Journal of General Physiology.* Part of it has already appeared in the *Journal of Neurophysiology* for July, 1941.)

Chemical composition of mitochondria and secretory granules. Albert Claude.

(This material is scheduled to appear in Symposia on Quantitative Biology, Cold Spring Harbor, Vol. 9, October, 1941.)

Native proteins and the structure of cytoplasm. Dorothy Wrinch.

General Scientific Meetings

August 26

Further studies of metamorphosis of ascidian larvae. Caswell Grave.

The mechanism of metamorphosis in the larva of *Cynthia partita* and in the larva of *Amaroucium constellatum* does not differ significantly from that in larvae of *Ascidia nigra* and of species of *Polyandrocarpa.*

Larvae of all of these species are induced to metamorphose very soon after hatching from the egg, or after they have been liberated from the brood pouch of the parent, by treatment with aqueous solutions of $CuCl_2$ in concentrations of the order of about 5×10^{-4} molar.

Observations of large numbers of larvae of *Cynthia* show that the normal duration of its free-swimming life varies between about 9 and 100 hours when segregated in vials containing 10 cc. of sea water, but that similar groups are induced to metamorphose within 2 hours when treated immediately after hatching with a 7×10^{-4} molar solution of $CuCl_2$.

The *Amaroucium* larva has a short free-swimming life of about 100 minutes maximum duration. This period is reduced to 40 minutes by treatment of larvae with a 1×10^{-4} molar solution of $CuCl_2$. Its mechanism of metamorphosis is even

more sensitive to an aqueous extract of the tissues of the adult *Amaroucium* zoöids. Groups of larvae treated with such an extract are induced to metamorphose within 20 minutes or about one-fifth of the normal period of larval life. Ascidian tissues are known to contain copper.

It is assumed that the copper salt may act as an enzyme poison, inhibiting an enzyme system of larval metabolism, thus ending larval life and liberating the adult action system, with its lower rate of metabolism, from the inhibition imposed upon it by the higher metabolic rate of the larval action system.

The "eye-spot" and light-responses of the larva of Cynthia partita. Caswell Grave.

The "eye-spot" of the *Cynthia* larva is degenerate and the responses the larva makes to light are correspondingly deficient.

The eye consists of a single defective, opaque lens and a small number of retinal cells that are wholly devoid of pigment granules and are not arranged to form an optic cup.

Cynthia larvae soon after hatching swim to the water surface in negative orientation to gravity but they show no orientation of their swimming movements to light. There is no persistent aggregation of the actively swimming larvae either at the most illuminated edge of the container or at the least illuminated edge. The larvae at all times tend to take an even distribution over the water surface.

However, if a lot of larvae are placed in a rectangular container (Leitz filter cell) enclosed in a box from which light is excluded except for a small opening at one end, the larvae after an interval of several minutes will be found to have moved to the least illuminated end of the cell. The same negative response to the directive beam will be found to have occurred as often as the cell is reversed in the box.

The mechanism by which larvae of the *Ammaroucium* type orient their swimming movements with reference to a source of light involves an eye with a relatively deep, pigmented optic cup into which light is concentrated intermittently at each rotation of the body of the actively swimming larva, by a system of three lenses. The *Cynthia larva*, lacking both optic cup and functional lenses, is deficient in an orientation mechanism. It may be partially compensated for by placing the larva in the path of a beam of directive rays of light.

Regeneration in the early zoöid of Amaroucium constellatum. Lloyd Birmingham.

Oözoids 1–8 days old were cut in one of three regions: (*a*) just below the pharynx, separating pharynx, atria, and hindgut in the anterior fragment and leaving stomach, gut, epicardium and heart in the posterior fragment, (*b*) through middle of pharynx leaving mainly pharyngeal and atrial tissues in the anterior fragment, and (*c*) just anterior to the heart leaving only the heart and part of the epicardium in the posterior fragment. The criterion of successful regeneration was the development of the beating heart.

Each fragment after operation (*a*) had about the same potentialities. Some 65 per cent of all fragments regenerated. About 70 per cent of fragments regenerating were members of the pair from one individual. The frequency of regeneration of anterior fragments was approximately the same as that of posterior.

Operation (*b*) showed a high frequency of regeneration in posterior fragments and intermediate frequency in anterior fragments. Only rarely did the posterior fragment from operation (*c*) regenerate; the anterior portion nearly always regenerated.

The time required for the development of a beating heart varied between 1 and 7 days. Average time was two days. The smaller the fragment relative to the total size of the individual the longer the time required.

The removal of the tail or adhesive papillae of the tadpole leads not to regeneration but to metamorphosis. Such treatment seems to hasten the breakdown of the larval action system allowing the adult action system to take over. Thus, regeneration of larval tissues has not been demonstrated; however, regeneration in the adult is of a complex type. Both tissue and organ regeneration take place. The latter requires the action of both totipotent cells and factors promoting proper differentiation.

Normal asexual reproduction of zoöids occurs 15–20 days after metamorphosis of the larva, yet the capacity for perfect regeneration is already present in such zoöids the day following metamorphosis.

Characteristics of the acceleration of Arbacia egg cleavage in hypotonic sea water. Ivor Cornman.

The acceleration, as previously reported, is a response of the egg to dilutions of sea water down to a concentration 88 per cent that of normal sea water. Acceleration of the first cleavage can be produced by beginning the treatment any time from a few minutes after insemination until at least as late as prophase of the first cleavage, and acceleration of the second cleavage by treating after the first cleavage. While the acceleration can result from exposure beginning during the mitotic cycle, a direct effect upon some phase of mitosis is not as yet demonstrated. Rather, evidence so far obtained favors the supposition of an indirect action, perhaps like a stimulus. (1) There is no clearly defined optimum when the acceleration is obtained by a range of dilutions. The effect resembles an all-or-none response, rising to a plateau in sea water diluted to 98 per cent, and continuing only slightly diminished to 90 per cent, where it begins to drop toward a retardation. (2) The first or second cleavage can be accelerated, but not both in the same egg. (3) Eggs from different urchins vary, some responding well, others not at all. This variation shows no correlation with the natural concentration of the sea water. Were the effect of hypotonicity a direct one, altering the cytoplasm so as to facilitate some phase of mitosis, it would be consistent with a more sharply defined optimum response, an acceleration of both cleavages, and possibly some correlation between the concentration of sea water and responsiveness of the eggs. On the contrary, the evidence, while not conclusive, points to a system within the egg which reacts to the full extent of its responsiveness to any dilution not great enough to interfere with cleavage, and which does not react in eggs which have been once stimulated, or for some natural reason lack the necessary energy or irritability.

Maternal inheritance in echinoderm hybrids. Ethel Browne Harvey.

Three different echinoderm hybrids have been studied, the California sea-urchins, *Strongylocentrotus purpuratus* ♀ × *S. franciscanus* ♂, the Woods Hole and Maine sea-urchins, *Arbacia punctulata* ♀ × *Strongylocentrotus dröbachiensis* ♂ and the Woods Hole sea-urchin and sand dollar, *Arbacia punctulata* ♀ × *Echinarachnius parma* ♂. In all three crosses, there is a marked maternal inheritance. The rate of development of the hybrid egg is that of the normal egg, and the size, shape, pigmentation and skeleton structure of the hybrid pluteus are like that of the mother with very little paternal influence. It has not been possible to obtain plutei from the reciprocal crosses, but Matsui, working at Woods Hole, found that "a cross between a female *Echinarachnius* and a male *Arbacia* . . . is in general purely maternal or nearly so, paternal characters in no case appearing" (1924, *Jour. Coll. Agr., Imperial Univ. Tokyo,* **7**: 211–236). Early larval development in these echinoderms therefore seems to be controlled by the cytoplasm rather than the nucleus. These experiments are preliminary to crossing the non-nucleate half-egg of one species, obtained by centrifuging, with the sperm of the other species, whereby conclusive evidence will be obtained as to cytoplasmic versus nuclear inheritance.

Intermediary carbohydrate metabolism of eggs and sperm of Arbacia punctulata before and after fertilization. E. S. Guzman Barron and J. M. Goldinger.

In 1928, Perlzweig and Barron found that the eggs of *Arbacia punctulata* contained carbohydrates and produced lactic acid; the lactic acid formation was increased when the oxidation was inhibited by HCN. The eggs of *Arbacia* contain also pyruvic acid (about 850 micrograms per gram dry weight). When lithium pyruvate was added to a suspension of eggs, the unfertilized eggs metabolized pyruvate at a rate of about 70 micrograms per hour per gram dry weight. The utilization of pyruvate increased five-fold after fertilization (354 micrograms per hour). Pyruvate metabolism is presumably catalyzed by diphosphothiamine, as in mammalian tissues and bacteria, for it is present in both fertilized and unfertilized eggs. The increased pyruvate metabolism after fertilization is not due to increased concentration of diphosphothiamine, because its concentration is not altered by fertilization (2.72 micrograms per cc. packed cells). The metabolism of pyruvate in the eggs does not proceed through the Szent-Györgyi cycle, because neither α-ketoglutarate nor succinate is oxidized. Addition of pyruvate depressed the O_2 consumption of fertilized eggs. The sperm of *Arbacia* also utilized pyruvate. In addition, the sperm oxidized α-ketoglutarate, succinate, and *l*(+)-glutamate, thus possessing all the elements for the operation of Szent-Györgyi's cycle for carbohydrate oxidation. The diphosphothiamine content of sperm was about twice that of eggs, 5.15 micrograms per cc. Whether this fivefold increase in the metabolism of pyruvate after fertilization is responsible for the increased O_2 uptake of the eggs on fertilization cannot be demonstrated by these experiments.

On metabolism of the heart of Venus mercenaria. A. E. Navez, J. D. Crawford, D. Benedict and A. B. DuBois.

In the study of the substrate(s) underlying the contraction of the heart of *Venus mercenaria* investigated by us (1940),[1] the following preliminary observations were made. The excised heart will keep for a long period its characteristic contractions when it is placed in a small quantity of aerated sea water. It may be whole, or "cut" in 3–5 pieces or "chopped" up in a large number of small pieces: the tissue remains highly contractile. Also for long periods this tissue (in any form or even in completely "minced" state) will respire at a uniform rate. Compared to O_2 consumption of the whole heart (100 per cent) the "cut" heart exceeds it by about 10 per cent, while the "chopped" heart is lower by about 10 per cent and the "minced" heart by about 50 per cent. But all seem to carry on the O_2 fixation with an R.Q. around 1.0. This applies to the heart unwashed in sea water. Often the washing (repeated from 1–6 times) of the pieces with sea water lowers the rate of this reaction. Addition of the washings brings back the rate around its normal value (some times a little lower, occasionally a little higher). The "minced" pulp can be centrifuged in two components, whose respiration rates are here given as percentage of the rate of the original "minced" pulp (100 per cent): (1) supernatant fluid: 30–35 per cent and (2) granular part: 65–70 per cent. Acetonic extracts and residues when reunited in water are inactive or inactivated.

In the study of the respiratory system we used the cytochrome-cytochrome oxidase-dehydrogenase system as a working hypothesis, in view of the presence of cytochrome C and succinic dehydrogenase in the heart (Ball and Meyerhof, 1940,[2] confirmed by us also). The addition of p-phenylenediamine determines a large

[1] Navez, A. E., Crawford, J. D., and Benedict, D., *Biol. Bull.*, **79**: 358, 1940.

[2] Ball, E. G., and Meyerhof, B., *Jour. Biol. Chem.*, **134**: 791, 1940.

increase in Qo_2 (up to 350 per cent) dépending on concentration. It persists for long periods. The poisoning of the heart by KCN (even at high concentration) determines an inhibition of 40 per cent at the most and the same in the presence or absence of p-phenylenediamine. The addition of succinate alone raises the Qo_2 by 10 per cent; succinate + methylene blue increase it to 150 per cent maximum, but this action is also elicited by M.B. alone and to the same extent. The inhibition of this reaction by KCN is as above 30–40 per cent. The other inhibitors tried gave markedly small or no effects: i.e. sodium fluoride, sodium azide, sodium iodoacetate, sodium selenite. Ethyl urethane alone has an enhancing effect (up to 20 per cent increase at concentration 0.1 per cent by weight). Definite indications (a strong lumiflavin reaction) point to the presence of a flavoprotein; a weak glutathione reaction is given also. In conclusion the simple working hypothesis does not fit the observational facts. Other experiments not reported here confirm this rejection. Additional observations are needed and are planned for the near future.

Coördination of ciliary movement in the Modiolus gill. Alfred M. Lucas and James Snedecor.

A study previously undertaken on this problem (*Jour. Morph.*, 1932, **53**: 243–276) employed moving pictures to record the waves of the lateral cilia of the *Modiolus* gill, but only wave-length could be satisfactorily determined and much time and film were used to obtain the data. The stroboscope employed in the present study has some advantages in that frequency and rate of wave propagation could be recorded: the wave-length was calculated. Even this method did not give sufficient number of records to allow critical analyses of data and some better procedure should be worked out for the problem.

A summary of results:

Temperature	Average frequency	Av. rate of wave propagation	Av. wave-length
° C.	*vib./sec.*	*μ/sec.*	*μ*
10	3.5	47	13.8
15	6.2	75	11.7
20	7.4	102	14.5
25	10.6	102	10.6
30	15.4	142	10.0
35	15.8	158	10.9
			Av. 11.9

The variation around the average was very great in every case so that the value of 11.9 μ for the wave-length is quite close to 13.1 μ obtained with moving pictures. Conduction of the coördination impulse in ciliated epithelium is similar to the conduction in nerves in that the wave-length is constant in both cases.

Preparing an animated diagram of somatic mitosis. Lorus J. Milne.

Although the factual basis for this study has been limited during the past year to the behavior of dividing epithelial cells from 25-mm. larvae of the salamander *Ambystoma tigrinum*, a much greater variation has been found than was anticipated. The timing of separation of daughter cells by formation of new cell membrane does not seem to be correlated with any given stage of the telophase transformations of the nucleus. It may be early before any alterations can be seen from the anaphasic condition of the chromosomes to very late, when the daughter nuclei are reorganized almost into interphase. Much variation is present in the dimensions of the spindle, the area of the metaphase plate and the volume changes

evident in the cytosome. Change in cell form has been followed in detail and observed to be polygonal in inter- and prophase, to become progressively more spherical in meta- and anaphase, and to recover the polygonal condition in late telophase or early interphase.

In technique a number of advances have been made, and an animation unit has been assembled from equipment provided by the Carnegie Corporation of N. Y. A very smooth S-curve was found to be the haversine relation such as given in tables of recent editions of Handbook of Chemistry and Physics (haversine $\theta = (1 - \cos\theta)/2$). This smooth curve was found excellent for transitions such as starts and stops of movements, rendering these completely free of "jump." Difficulty in applying ink and paint to cellophane was overcome by mixing the pigment solutions with 2 per cent honey and 8 per cent of 10 per cent Fotofoam, 90 per cent of water color. The honey keeps the pigment solution from drying out completely, hence it does not crack or peel off. Fotofoam, apparently a bile salt derivative, reduces the surface tension of the color and allows it to spread easily on the shiny cellophane. A dissolving shutter with both manual and automatic control has been developed, using the new Polaroid-H glass. The two uncrossed plates of this glass transmit about 50 per cent of incident light; crossing the axes to 85° cuts the transmission to about 1/400 of the 50 per cent value, while at 90° the transmitted light from bright sun through an F: 1.5 lens is photographically inactive to even the fastest films. The decrease in transmission is almost linear, and until crossed more than 86° seems uniform throughout the spectrum. Beyond that limit, the violet end is less restricted than the longer wave-lengths.

Stimulation by intense flashes of ultra-violet light. E. Newton Harvey.

Any effective stimulus must be of sufficient intensity and also change rapidly in intensity. In order to obtain high intensity ultra-violet light a three microfarad condenser discharge at 20,000 volts is passed through a quartz mercury vapor sterilamp, according to the method of Rentschler. A single discharge, lasting a few millionths of a second, is capable of immediately killing bacteria, disintegrating protozoa, stopping cyclosis, ciliary and amoeboid movement, contracting myonemes and suppressing bacterial luminescence. In *Nitella* the protoplasmic rotation is reversibly stopped, sometimes only on the side of the cell toward the ultra-violet or only in a portion of the cell covered with quartz, not in that region covered with glass. It was found that sometimes the ultra-violet light would start an action potential locally and sometimes the potential was propagated over the whole cell, showing that ultra-violet light can stimulate in the same manner as electrical stimuli. The stimulation of vertebrate muscle and nerve is not yet certain. None of the above effects ever occur when the cells are shielded from the discharge by glass.

The influence of the medium on the radiosensitivity of sperm. T. C. Evans, J. C. Slaughter, E. P. Little, and G. Failla.

The ability of sperm to fertilize eggs is affected by roentgen radiation. However, it has been found that: (1) Sperm irradiated in the seminal fluid in concentrated form are very radioresistant. (2) If the seminal fluid (containing sperm) is diluted with sea water and then irradiated, the sperm become progressively more radiosensitive with dilution. The increase of radiosensitivity in a certain range of dilution is inversely proportional to the concentration of the sperm. (3) Sperm in dilute suspensions can be made more resistant again by the addition, before irradiation, of small amounts of egg albumen, gelatin, *Arbacia* egg water, and glycylglycine. (4) Sperm, in sea water, made inactive (by centrifugation) are more radioresistant than those actively swimming about during the exposure to the roentgen radiation. (5) The actions possibly involve two stages: (1) some harmful agent is momentarily produced in the water, and (2) the activity of the sperm affects the amount of contact with the harmful agent.

The effect of roentgen radiation on the fertilizing capacity of the sperm may be regarded as an indirect effect which can be altered by changing the medium. A more direct action of the radiation (not easily modified by the medium) is the delay in cleavage observed when eggs are inseminated with irradiated sperm.

Comparative pharmacology of myogenic and neurogenic hearts. C. Ladd Prosser and George L. Zimmerman.

The hearts of mollusks and adult vertebrates are inhibited by acetylcholine. These are myogenic hearts. The hearts of decapod crustaceans of insects and of *Limulus* are accelerated by acetylcholine and are neurogenic.

Acetylcholine accelerates and raises the tonus of the hearts of *Arenicola cristata*. In high concentration it stops the heart in systole. The threshold is one part in 10^8 without eserine. Eserine potentiates and atropine antagonizes the action of acetylcholine. The dorsal vessel is accelerated by acetylcholine but higher concentrations are required. Small amounts of potassium added to sea water bathing the *Arenicola* heart accelerate and raise the tonus while small amounts of excess calcium slow the hearts. A pacemaker is located in the small vessel connecting the laterogastric vessel with the heart. Adrenalin accelerates the hearts and in high concentrations stops them in diastole. From the above results we postulate that the heart of *Arenicola* is neurogenic.

In the *Limulus* embryo the heart begins its beat myogenically on the twenty-first day. It becomes neurogenic at about the twenty-eighth to thirty-third day. During the myogenic period this heart is insensitive to acetylcholine (1 in 10^4) with or without eserine. Beginning from the thirty-first to thirty-fifth day the hearts are accelerated by acetylcholine.

The heart of *Daphnia* is inhibited by acetylcholine and in the heart of *Eubranchippus* there is no effect (as in early *Limulus* and in vertebrate embryos). The heart of *Talorchestia* is accelerated by acetylcholine.

It is suggested that those hearts which are accelerated by acetylcholine are neurogenic and that those which are inhibited or unaffected are myogenic.

Structures concerned with yolk absorption in Squalus acanthias. Lois E. TeWinkel.

In Balfour's monograph on the Development of Elasmobranch Fishes (1876) and in a paper by Beard (1896, *Anat. Anz.*, **12**: 334) it is stated that yolk, in the later embryonic stages of these fishes, passes bodily up the yolk stalk into an internal sac. This sac is an outgrowth of the stalk at its point of entrance to the intestine and yolk taken into it eventually reaches the alimentary canal, there to be digested.

Living *Squalus acanthias* embryos from 110–230 mm. in length and preserved specimens of 60 mm. have been studied. The internal yolk sac increases enormously in size between the 60 and 230 mm. stages. It is just clearly visible in gross dissections of the former, while, in the latter, the external sac has shrunk to an empty stub only 6 mm. in length and the internal sac is very large (approximately 45 mm. long and 18 mm. in diameter). The method by which yolk is transported to the internal sac has not yet been determined.

Sections show yolk platelets in the internal sac of a 60 mm. embryo but apparently not any are present in the intestine, whereas, in a 150 mm. specimen, the spiral valve region is filled with an emulsion of yolk in various stages of digestion. Cells of the simple, low volumnar epithelium lining the external yolk sac contain scattered fat droplets and glycogen in the 110 and 150 mm. specimens studied, indicating that, so long as the external sac is large and well vascularized, it plays some part in embryonic nutrition.

The distribution of elastic tissue in the arterial pathway to the carotid bodies in the adult dog. William H. F. Addison.

In the examination of many series of sections through the region comprising the bifurcation of the common carotid artery, the carotid sinus and the carotid body, there is found great uniformity in the structural tissues constituting the walls of the arterial vessels supplying blood to the carotid body tissue. But, as is frequent in the vascular system, the arrangement of the vessels may present many variations. In the case here reported, from the right side of an adult dog, the most striking variation is that the carotid body is not aggregated into one large mass but is distributed along the usual arterial pathway as several separate masses.

The blood supply to the carotid body in the dog is from the occipital artery, which in this animal is the first branch of the external carotid artery above the bifurcation of the common carotid artery. The occipital artery arises at a variable distance from the bifurcation and sometimes from the bifurcation itself. In the present case there is an interval of 3 mm. between the bifurcation and the origin of the occipital artery. The structure of the walls of the beginning is different from that of the rest of the occipital artery, inasmuch as it is elastic-walled, non-muscular, similar in structure to the carotid sinus. Because of its structure and its wider diameter than the rest of the occipital artery, this beginning part of the occipital artery may be called the occipital sinus. The further course of the pathway to the carotid body is as follows. From the occipital sinus is given off a short branch which is at first elastic-walled. This branch soon divides into two sub-branches, of which one has muscular walls and the other has elastic-tissue walls. The latter gives off the blood supply to the carotid body and then becomes muscular in character.

In the present case this arrangement of the elastic-walled vessels is present, but the carotid tissue is distributed at intervals alongside the carotid sinus, its elastic-walled branch and the latter's elastic-walled sub-branch. From each of these parts of the arterial pathway little vessels come off to supply the separate masses of carotid tissue, and in each little vessel the wall is elastic in structure.

Thus, in this case where the carotid body tissue is divided into small portions, each portion is still provided with blood through an elastic-walled non-muscular series of channels, while the continuation of these vessels, except those terminating in the carotid tissue, is always muscular, and under the control of the vasomotor system.

Behavior of the arterioles in hypertensive rabbits, and in normal rabbits following injections of angiotonin. Richard G. Abell and Irvine H. Page.

It is well known that in hypertensive patients, and in animals made hypertensive experimentally, there is an increase in resistance to blood flow. Although it has been observed that the retinal arterioles of hypertensive individuals are narrower than normal, there are no reports of measurements of their diameters before, as well as after the development of hypertension.

In the present experiments, living arterioles in transparent moat chambers in ears of normal rabbits were observed with the microscope, and their diameters measured. The animals were then made hypertensive either by the method of Goldblatt (1934) or the method of Page (1939, 1940), and the diameters of the same arterioles measured again.

Of the 7 operated rabbits, 4 became hypertensive. The blood pressure rose to from 1.4 to 2.1 times the normal level. The arterioles constricted from approximately 0.4 to 0.7 their control diameters when the rabbits became hypertensive.

No constriction occurred in those rabbits which did not become hypertensive.

The capillaries and venules did not constrict in any of the experiments.

In order to see whether the arteriolar constriction that occurred in the hypertensive rabbits might have been due to angiotonin, 0.2 cc. of angiotonin was injected intravenously into a normal rabbit, and the resulting arteriolar constriction measured. After the arterioles had returned to their control diameters, the rabbit was made hypertensive, and the resulting constriction of the same arterioles measured.

In both instances the arterioles constricted to approximately 0.5 their original diameters, and the blood pressure rose to about 1.4 times its control level.

This suggests that the arteriolar constriction that occurred in these hypertensive rabbits might have been due to angiotonin.

It should be emphasized that these studies have been made on vessels in the ears of rabbits, which are notoriously active in dilatation and constriction; consequently the *extent* of constriction found here should not be applied to other peripheral arterioles without further study.

AUGUST 27

Catalysis of ionic exchanges by bicarbonates. M. H. Jacobs and Dorothy R. Stewart.

The acceleration of hemolysis in solutions of NH_4Cl by low concentrations of bicarbonates, first observed by Ørskov, was explained by Jacobs and Parpart as essentially a catalysis of diffusion involving entrance of CO_2 and NH_3 into the cell, conversion of CO_2 into HCO_3^-, exchange of HCO_3^- for Cl^-, reconversion of HCO_3^- into CO_2, and so on. This principle can be extended to other ionic exchanges in the erythrocyte in which ammonium salts are not concerned. In general, as long as the necessary pH differences exist between the anion-permeable cell and its surroundings, and anions such as Cl^- are available for exchange, a reaction which for brevity may be represented as $HCO_3^- \rightleftharpoons CO_2 + OH^-$ may take place in opposite directions on the two sides of the membrane, the resulting cycle leading to the final equilibrium distribution of ions far more rapidly than is possible in the absence of bicarbonates. This catalysis-like effect is illustrated by the volume changes of erythrocytes that occur on changing the reaction of the surrounding medium or on suspending the cells in a solution of a salt with a bivalent anion such as SO_4^{--}. With certain limitations and qualifications, changes in the amounts of bicarbonates in the solution affect only the rate of the process and not the position of the final equilibrium.

The rôle of carbonic anhydrase in the catalysis of ionic exchanges by bicarbonates. Dorothy R. Stewart and M. H. Jacobs.

The theory suggested in the preceding abstract for the catalytic effect of bicarbonates on the attainment of certain ionic equilibria involves the reversible reactions

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO^-.$$

The first of these reactions is known to be strongly accelerated in both directions by the enzyme carbonic anhydrase. Considerable support is therefore given to the theory by the observation that the catalysis-like effect of bicarbonates on ionic exchanges in the erythrocyte is in turn strikingly influenced by this enzyme. The importance of carbonic anhydrase in such processes can be shown either by adding it to the medium in which the cells are suspended or by inhibiting its action within the cells by means of sulfanilamide or cyanides. In general, under the conditions of these experiments, the enzyme is more effective inside than outside the cells, but under certain circumstances its external effect may also be very striking. Inhibition of the enzyme within the cell by cyanides occurs almost instantly, that by sulfanilamide reaches its maximum only after several minutes. Both effects may readily be reversed by washing the cells.

Some effects of desoxycortico-sterone and related compounds on the mammalian red cell. Martin G. Netsky and M. H. Jacobs.

The sodium salt of the phosphate ester of desoxycortico-sterone (Na DOC PO_4) was found to produce sphering of human erythrocytes in concentrations as low as 1 mg. per cent or 2×10^{-4} mols per liter. The sodium salt of the phosphate ester of di-desoxycortico-sterone (Na di DOC PO_4) produced sphering at 0.4 mg. per cent or 5×10^{-7} mols per liter; 21-sodium hydrogen phosphate of 3-acetoxy-Δ^5-pregnene-21-ol-20-on (Na AcO pregnene PO_4) also produced sphering, but sodium glucuronidate pregnanediol did not. Neither compound E, nor free desoxycortico-sterone, nor Kendall's amorphous fraction gave sphering. Sphering ability seems to be associated with molecules of polar: non-polar structure, although a special form of non-polarity is required. Sphering can be reversed or inhibited by the addition of serum protein, the amount of protein necessary being the same in either case. The reaction is quantitative, 1 mg. of either Na DOC PO_4 or Na di DOC PO_4 being equivalent to approximately 10 mg. of serum protein. In higher concentrations, those substances which produce sphering are directly hemolytic. Direct hemolysis is also prevented by the addition of serum protein, and apparently the same type of chemical structure is required for it as for sphering. The effect of some of these substances on permeability to ammonium chloride and to glycerol was studied by the hemolysis method. In the case of ammonium chloride, the hydroxyl-chloride ion exchange was inhibited and hemolysis slowed markedly, both in human and beef cells. The effect on glycerol hemolysis of beef cells was a decrease in the time of hemolysis at all pH levels. For human cells this was also true at pH levels more acid than about 6.8, but at higher pH values, the effect was a more complicated one, low concentrations increasing the time, higher ones decreasing it below the level of the control. For the three Na PO_4 salts, the order of activity was: Na di DOC PO_4 > Na DOC PO_4 > Na AcO pregnene PO_4. Similar effects on glycerol permeability, obtained with compound E and Kendall's amorphous fraction, indicate that an extremely polar: non-polar structure is not required for permeability changes.

Permeability of the Arbacia egg to potassium.[1] Herbert Shapiro and Hugh Davson.

The permeability of the *Arbacia punctulata* egg to ions, and the problem of the maintenance of concentration gradients in this cell, have hitherto not been investigated. Chemical analyses were made of the potassium content of the egg and of sea water. The eggs were found to have approximately twenty times as much potassium as the sea water. Fertilized eggs contained very nearly the same amount of potassium as unfertilized. Suspensions of eggs were placed in a shallow layer on the bottom of Erlenmeyer flasks, and oxygenated by passing moistened oxygen over the suspensions. The flasks were immersed in a thermostat maintained at 25.6° C. Samples of suspension were taken at regular intervals, centrifuged, and the supernatant fluid analyzed chemically for potassium content. Runs were made over periods varying from two to seven hours. Total cell volume was determined by measurement of cell diameters, and of cell concentration by a dilution method. Both unfertilized and fertilized eggs lost potassium on coming in contact with sea water, though at a slow rate; from about 1.5 to 8 per cent of the cellular potassium diffused out in a two-hour period. Eggs placed in nitrogen also lost potassium, though at a rate not markedly different from that of eggs in oxygen. Eggs in artificial sea water with five times the normal potassium content accumulated potassium, and did this against a gradient. When placed in artificial sea water

[1] This investigation has been aided by a grant from the Penrose Fund of The American Philosophical Society.

containing four times the normal amount of calcium, fertilized eggs lost potassium more rapidly than those in normal sea water. When the excess calcium sea water was replaced by normal sea water, potassium began to reenter the cell, once more against a gradient.

Lipo-protein complexes in the egg of Arbacia. A. K. Parpart.

Determinations of the total lipid were made on "lyophylled" eggs of *Arbacia*. Values of total lipid were 5.4 per cent of the whole egg or 26.9 per cent of the solids of the egg. By methods found applicable to erythrocytes it was found that 77 per cent of the total lipid behaves as though it were bound to protein.

Eggs exposed to a 30 parts NH_4Cl, 70 parts sea water mixture for four to ten hours showed no decrease in the amount of lipid bound to protein. Eggs undergoing development for five hours did not change in total lipid or in lipid bound to protein.

These data suggest that the major portion of the lipid acts as a structural component of the egg cell more than as a metabolic component.

The relation between protoplasmic streaming and the action potential in Nitella and Chara. Samuel E. Hill.

Much past work has shown that protoplasmic streaming is profoundly affected by passage of electrical currents through the cell, or by passage of action currents along the cell. Does presence or absence of streaming bear any relation to electrical irritability?

Under certain conditions, for example, soaking for three days in distilled water, *Nitella* cells lose their ability to give an electrical response to stimulation, yet the protoplasmic streaming continues. Streaming is thus not a sign of irritability.

The streaming may be stopped by very weak electrical stimulation. If the applied voltages are small enough, the streaming slows down and gradually comes to a stop, no action current appearing. If, however, a larger stimulating voltage is applied, an action current appears and the streaming stops abruptly. After 30 to 60 seconds the streaming begins anew, starting and stopping abruptly several times as if pulling against high viscosity. After a few seconds of this the streaming again becomes regular. These abrupt starts and stops are accompanied by no electrical changes. During the time while the protoplasm is at a standstill, it is possible to provoke an action current by electrical stimulation. This can be repeated at intervals (about 25 seconds) for 30 minutes or more, every stimulation being followed by an action current and the protoplasm at a standstill all the while. After a rest of a few minutes the streaming begins again.

The streaming appears to have no antecedent relation to the action current, since presence of streaming does not indicate electrical irritability, nor absence of streaming indicate failure of electrical response.

Observations on luminescence in Mnemiopsis. Aurin M. Chase.

Harvey and Korr (*J. Cell. Comp. Physiol.*, 1938) found that extracts or fragments of the photogenic cells of *Mnemiopsis leidyi* can luminesce even in the complete absence of oxygen. Under such conditions continuous luminescence occurs rather than the brief flashes characteristic of the living organism; an indication that nervous control of the process has disappeared.

The present experiments concern luminescence of the intact, living animal. Upon electrical stimulation through the sea water flashing occurs along the rows of swimming plates. After complete de-aeration (thirty to forty minutes flushing with purified hydrogen) no flashing can be elicited by electrical or mechanical stimulation. Three to five minutes after re-admitting air the animal again responds.

The cycle of de-aeration and re-aeration can be repeated as many as three times before the animal dies. Movement of the swimming plates stops at about the same time that luminescence on electrical stimulation ceases. As the animal begins to disintegrate, either in an atmosphere of air *or of hydrogen*, a dim, continuous luminescence gradually appears along the rows of swimming plates. This lasts for about an hour, and undoubtedly represents the basic luminescent reaction as studied by Harvey and Korr, freed from its normal nervous control.

Mnemiopsis in sea water (air present) loses its ability to luminesce on electrical stimulation within 15 seconds after addition of 0.0001 *M* KCN, although the swimming plates continue to move for ten to twenty minutes. Eserine (1:2,000) increases the sensitivity to luminesce on mechanical stimulation and also increases the duration of the luminescent flashes. Addition of acetylcholine (1:3,000) enhances this effect. Returning the animals to plain sea water gradually restores the normal response. Adrenaline (1:100,000) apparently decreases the sensitivity but the effect is less clear-cut than the increased sensitivity caused by eserin and acetylcholine.

Papers Read by Title

Photodynamic studies on Arbacia eggs. Fred W. Alsup.

Rose bengal in concentrations of 1 part dye to 20,000 or more parts sea water and eosin in a concentration of 1:2,000 have no effect, in the dark, on the relative viscosity of the inner protoplasm of the unfertilized eggs of *Arbacia punctulata*. However, when the eggs are exposed in either of these dye solutions to light from a 1000-watt electric bulb, i.e., exposed to photodynamic action, the viscosity is markedly increased. On the average, the viscosity of eggs exposed to photodynamic action is about 40 per cent higher than that of unexposed eggs. Increases in viscosity can be detected within 5 seconds after the exposures and reach a maximum in about 1–5 minutes after the exposures.

The unfertilized eggs of *Arbacia* become activated when exposed to photodynamic action. Most of them show marked amoeboid movement and do not cleave when left in the dye solutions or removed to sea water. A very low percentage of these eggs divide in an irregular fashion. If eggs are removed to mixtures of sea water and isotonic calcium chloride soon after they are exposed to photodynamic action, a much higher percentage cleave irregularly. Apparently calcium strengthens the cortex of the eggs, which has been liquefied by photodynamic action.

Disruption of mitosis in Colchicum *by means of colchicine.* Ivor Cornman.

Colchicum, the commercial source of colchicine, contains a concentration of the alkaloid sufficient to block mitosis in other plants. By growing excised roots of *Colchicum* corms as temporary cultures in small vials, it has been possible to test a wide range of conditions upon uniform material. During the 8½ hours of the experiments, mitosis continued normal in tap water, in 1 per cent colchicine, and in sucrose isomolar with the effective colchicine concentrations. Mitosis was blocked in *C. byzantinum* by 5 per cent colchicine and in *C. autumnale* by 10 per cent. The cytological picture in *C. byzantinum* is typical of colchicine effects: disappearance of the spindle structure and then on the spindle material; accumulation of blocked metaphases; inhibition of the cell plate; appearance of tetraploid and binucleate cells. Upon removal from the colchicine, the cells recovered and continued dividing. They appeared normal except for the abnormal orientation of the spindles. Onion roots cultured in the same way showed typical colchicine effects in 0.01 per cent colchicine. It is concluded that the spindle mechanism in

Colchicum is the same as in other angiosperms with regard to the colchicine effect, and that the immunity of *Colchicum* to its own alkaloid resides in some extra-mitotic protective mechanism.

The effect of roentgen radiation on the jelly of the Nereis zygote. T. C. Evans.

Several investigators have reported that irradiation of *Nereis* ova results in an extreme swelling of the fertilization membrane following insemination. The swelling of the fertilization membrane is related to the amount of jelly retained within the membrane. Within a few minutes after insemination, unirradiated eggs will exude the jelly rapidly and it will pass through the membrane to surround the zygote with a thick viscous layer.

In this investigation zygotes have been irradiated after the jelly has been exuded, and it has been found that the radiation disperses the jelly immediately. The dosage required is about 44,000 roentgens.

The action of the roentgen radiation on the jelly, after it has been produced externally, is similar to that previously reported for the jelly of *Arbacia* and *Asterias* eggs.

Tests of nucleoli and cytoplasmic granules in marine eggs. R. Ruggles Gates.

In testing the nucleoli of the eggs of *Asterias, Arbacia, Mactra* and *Chaetopterus* for phospholipids the absence of lipoids and phospholipids from these bodies was shown by negative tests with Sudan III, osmic acid, Scharlach R and Feulgen without hydrolysis. In *Mactra* eggs treated with chloroform the nucleolus was unchanged. Observation of fresh eggs of these genera in sea water under an immersion lens showed that the nucleolus consists of two parts, like immiscible fluids, one enclosed within the other. The outer part is more quickly soluble in tap water than the inner portion.

While the nucleus is unaffected when Feulgen is added without previous hydrolysis, the cytoplasm in all these eggs unexpectedly showed a gradually deepening magenta color, indicating the presence of substances which have a free aldehyde group. The same reaction was obtained with the muscles of *Chaetopterus.* The cytoplasmic substances are relatively insoluble in water but more soluble in alcohol. They appear to belong to the acetalphosphatids of Feulgen and Voit. *Fucus* eggs and oögonia showed no color change at ordinary temperatures, so these substances may be characteristic of animal cells; but on exposure to air or rise of temperature a pink color develops in certain tissues of the *Fucus* thallus in Feulgen.

When the cytoplasm of *Chaetopterus* eggs is examined under an immersion lens after the Feulgen reaction, many of the granules, both large and small, are magenta in color. In crushed eggs, some granules are seen to be deep magenta, some pale, some uncolored, and there is a diffuse pink in the cytoplasm. The smallest granules are most intensely colored, some granules of all sizes remaining uncolored.

Sex-linkage of stubby (sb) in Habrobracon. Russell P. Hager.

Linkage of fused (fu) with the sex alleles has been demonstrated to occur with crossing-over in 8.6 per cent (Speicher) to 17.6 per cent (Bostian) of the cases (*cf.* Whiting, P. W., *J. Morph.*, **66**: 323–355). Since stubby (sb) was known to be linked with fused, tests were made to determine the percentages of crossing-over between stubby and fused, and stubby and the sex alleles so that the order of the factors could be mapped. Females heterozygous for stubby and fused (sb/fu) yielded 479 sb, 507 fu, 136 sb fu, and 179 + haploid males. Crossing-over between

stubby and fused is therefore about 24.2 per cent as data collected previously by others have indicated.

Orange-eyed females heterozygous for stubby were mated to stubby males:—o sb xa/o xb × sb xa or sb xb. The crosses with sb xa yielded 370 (linked) heterozygous and 228 (cross-over) stubby females; 10 (cross-over) heterozygous and 32 (linked) stubby diploid males. The crosses with sb xb yielded 642 (cross-over) heterozygous and 1132 (linked) stubby females; 70 (linked) heterozygous and 57 (cross-over) stubby diploid males. The cross-over class among the biparental males is of the type opposite to the cross-over class among the females. The cross-over percentage was calculated by extracting the square root of the products of the comparable classes. $\sqrt{642 \times 228}/(\sqrt{642 \times 228} + \sqrt{1132 \times 370})$. Crossing-over between stubby and the sex factor was accordingly 37.5 per cent among the females and 33.6 per cent among the diploid males.

The order of factors is therefore:

sex—(8.6 per cent to 17.6 per cent)—fused—(22 per cent to 24.2 per cent) stubby: sex.....(33.6 per cent to 37.5 per cent).....stubby.

The elasmobranch interrenal; a preliminary note. The interrenal body of Alopias vulpinus (Bonnaterre). E. R. Hayes.

This description is based upon the examination of a thirteen-foot "thresher" shark, *A. vulpinus*. The interrenal is an unpaired, elongate, yellow body lying between the caudal portions of the two kidneys and immediately dorsal to the posterior cardinal sinus. Slightly asymmetric in position, the gland is more closely applied to the left kidney. Beginning 5 cm. from the posterior limit of the kidney, it extends craniad 30 cm. and is discontinuous at one point in its anterior half. In cross-section, the gland is roughly oval, the greatest diameter ranging from 8–10 mm. posteriorly and tapering to 2–3 mm. anteriorly.

Microscopically, a rather thin capsule is seen covering a strikingly uniform parenchyma which shows no lobulation. It consists of cords of lipid-laden cells interlacing with blood sinuses and clothed by the endothelium lining the sinuses. It is possible to distinguish only one type of cell in the parenchyma. This type closely resembles the "spongiocytes" of the mammalian adrenal cortex. The cells are heavily packed with droplets of Sudanophil lipid which also blacken with OsO_4 (labile in xylol) and with the Schultz test give a strongly positive reaction for cholesterol. Paralleling the uniformity of these reactions is the even distribution throughout the gland of a considerable amount of birefringent material. Certain cells show evidence of nuclear pycnosis although in other respects they are indistinguishable from neighboring cells. The life history of the cells of this gland remains to be worked out.

The cytology of Amoeba verrucosa. Dwight L. Hopkins.

Amoeba verrucosa is a fan-shaped form typified by longitudinal folds and grooves which are formed continuously on its superior surface. It feeds on a variety of plant and animal organisms including bacteria and rotifers. In feeding it flows over the prey, trapping it in a groove, or depression in the under-surface. Once the prey is trapped, the sides of the groove are extended downward and under until they meet and the victim is enclosed in an irregular tube. The folds or sides of the groove, when they meet, form closely approximating, irregular lines, which remain visible for some time. The tube is not closed completely. When the prey is rejected as food it is squeezed out from the posterior end of the tube; when accepted, only the water is squeezed out and the food is drawn into the interior. The food vacuole thus formed contains little or no fluid.

Once the food vacuole is inside, small neutral red staining vacuoles which are very numerous in the cytoplasm are attracted to its walls and enter by coalescence. This process renders the recently formed food vacuole stainable with neutral red. As digestion proceeds the color is first red, then yellow, and finally colorless, just before the vacuole is expelled. Due to the addition of neutral red vacuoles, food vacuoles during digestion may contain considerable fluid material, but most of this is absorbed by the cytoplasm before the residues are expelled.

Contractile vacuoles arise by coalescence and swelling of smaller vacuoles. The cytoplasm contains numerous small rod-shaped and spherical bodies which come to the region where contractile vacuoles form through the plasmagel and when the gel transforms into sol, they cluster around the old contractile vacuole. These small bodies, as well as the membrane of the contractile vacuole, take Janus Green B faintly. The small bodies which stain finally with Janus Green B form small contractile vacuoles by coalescence and swelling.

Observations on the melanophore control of the cunner Tautogolabrus adspersus (Walbaum). George W. Hunter, III, and Edward Wasserman.

Background responses under a constant source of illumination were studied in the cunner, *Tautogolabrus adspersus*. Black-adapted fish responded to a white background in 15 seconds, reaching a maximum in 50 minutes, while white-adapted fish placed in a black background required an average of about ten seconds for the first phase of the reaction and 50 minutes to complete the response. Responses of white- and black-adapted fish to yellow and blue backgrounds were intermediate to the controls on white and black backgrounds. Placing cunners in complete darkness produced paler fish but darker than the white-adapted ones, while enucleated cunners gave no response to background changes. Both experiments indicate the importance of the eye in normal responses to background changes.

Cutting of caudal fin rays and the accompanying nerves of white-adapted fish produced dark bands due to expanded melanophores in 40–45 seconds and a maximum response in 45–60 minutes. Windows gave similar results. Cuts proximal to the band produced darkening of the freshly cut area distally as far as the original cut. Electrical stimulation of the medulla and roof of the mouth at the lowest frequency possible on a Harvard inductorium using 6 volts, produced a temporary darkening of white-adapted fish lasting about a minute, while the highest frequency gave a partial but distinct blanching of black-adapted fish.

Observations on recutting of fading bands and the fading time of single and multiple fin ray cuts are still being carried on. Experiments on hypophysectomized fish as well as the effects of drugs and salts on melanophores are in progress and will be reported elsewhere.

The evidence accumulated thus far suggests that this northern representative of the wrasses, the cunner, has a melanophore system controlled by adrenergic and cholinergic sets of nerve fibers. While the pituitary has given a positive test for intermedin when injected into a blanched frog, its rôle in the normal control of melanophores has not yet been determined.

The control of melanophores in the cunner is being studied as one phase of the problem dealing with pigment production and its control about the cysts of the trematode metacercaria, *Cryptocotyle lingua*, which occur on the scales of the cunner.

The influence of temperature on reconstitution in Tubularia. Florence Moog.

The fact that the body size of Metazoa is generally greater at lower temperatures has been noted frequently. In *Tubularia* the size of the reconstituted

hydranths is similarly affected by low temperature. The table shows the length of the reconstituents and the time for their formation in one experiment consisting of batches of 25 sections of stems, each 6 mm. long, kept in 200 cc. of sea water.

Temperature	Time to constriction of primordium	Length of primordium
° C.	*hours*	*micra*
22.2	44.7	854
19.0	55.1	918
14.0	59.5	1050

In three experiments the reconstituents at 14° averaged 15.2 per cent longer than at 22°, and their time of formation was 39.3 per cent longer.

Some authors have pointed out, on the basis of studies on embryonic and adult vertebrates, that the larger body size might be accounted for by the fact that less food is needed to maintain tissues at low temperatures, so that more can be used in building new protoplasm. But since an external food supply is not a factor in reconstitution of *Tubularia,* it seems likely that the increased size is due at least in part to modification of chemical equilibria. Low temperature would most likely slow the chemical processes involved in the conversion of tissue, without markedly affecting diffusion, so that the change in size might result from the deeper penetration of oxygen.

It is interesting to note that low temperature increases the size of the reconstituted hydranth while decreasing its rate of formation. Other agents, such as low oxygen concentration, low pH, cyanide, azide, and urethanes, which decrease the rate, decrease the size also. Evidently the processes underlying reconstitution velocity and determination of size of the primordium are independent to a considerable degree.

Factors influencing the pigmentation of regenerating scales on the ventral surface of the summer flounder. Clinton M. Osborn.

From summer flounders which had been black- or white-adapted, scales were plucked in a definite pattern from the naturally white lower surface and the fishes returned to their original tanks. It was apparent within two weeks that the regenerating scales on the white-adapted flounders were white, while those on black-adapted fishes developed melanophores. When black-adapted flounders were blinded by enucleation, the regenerated scales were pigmented regardless of the shade of the background, indicating that intact eyes were not essential to pigment production.

To test the direct effect of light, flounders prepared in three different ways were illuminated strongly underneath through special glass-bottomed aquaria. In one experiment white-adapted fishes were illuminated ventrally. The regenerated scales were *white.* In the next experiment the fishes were black-adapted (black walls and ceiling) while ventrally illuminated and grew melaninated scales. Finally, blinded fishes (in the dark phase, but not maximally black) regenerated pigmented scales when ventrally illuminated. The results from these three experiments were qualitatively similar to the original observations, indicating that bright illumination had little influence on the color of the regenerating scales. This was further substantiated in experiments where the regenerated scales were melaninated on black-adapted fishes in tanks dimly lighted during the day and totally dark at night.

It is concluded that the color of scales regenerating on the naturally white lower surface of the summer flounder is influenced primarily by the physiological factors (nervous and endocrine) which cause the upper surface of the fish to assume the pale or the dark phase. Factors which produce the dark phase favor

the development of melanophores in regenerating ventral scales while physiological agents causing the pale phase allow white scales to regenerate. Light appears to have little direct effect on the color of regenerating scales although melanination is somewhat accelerated by light in physiological conditions which favor pigmentation.

Hypersensitization of catfish melanophores to adrenaline by denervation. G. H. Parker.

The melanophores in a denervated caudal band of a catfish of intermediate tint will concentrate their pigment after the fish has received 0.008 or even 0.004 of a milligram of adrenaline per 100 grams of fish. These dosages do not induce noticeable pigment changes in the innervated melanophores of the rest of the fish. Weaker doses of adrenaline have no obvious effect on either denervated or innervated melanophores. Stronger doses induce pigment concentration in both denervated and innervated melanophores. The greater sensitivity for adrenaline thus shown by the denervated melanophores as compared with the innervated ones may be due to the fact that, after the color nerves have been cut, not only the adrenergic but the cholinergic fibers degenerate. Consequently the injected adrenaline does not find in the denervated bands the local dispersing agent acetyl choline for an opponent as it does in the innervated regions. Hence in denervated bands adrenaline is able to act efficiently at lower concentrations than in innervated areas. There is no reason to suppose that denervation alters the melanophores themselves. Denervation in the catfish appears merely to remove an adrenaline opponent and thus to give this agent more effective sway. This explanation of melanophore hypersensitization to adrenaline may not apply to other instances of the special sensitization of effectors, but it appears to meet the requirements in the melanophores of the catfish.

Implants consisting of young buds, formed in anterior regeneration in Clymenella, plus the nerve cord of the adjacent old part. Leonard P. Sayles.

Worms were cut to regenerate anteriorly. After 2 to 9 days, any newly-formed material plus the adjacent anterior nerve cord was inserted into the thirteenth segments of hosts. In some cases the original buds dropped off. Then either (1) no material formed at the implant or (2) small new buds, each terminating in the dorsal half of an anal segment, developed. All of the latter terminated ventrally in truncate regions. These results were similar to those obtained when pieces of anterior nerve cord from non-regenerates were inserted at posterior levels of hosts.

When the original bud was retained, the results were modified. When only a small blastema had regenerated, the implant gave rise, in many cases, to a bud terminating in a partial anal segment dorsally and either a large, conical mass or a weakly developed peristomium ventrally. When a small, 3-day type head was present, this bud might continue to develop. Frequently, however, a partial anal segment appeared on the dorsal side of the bud. Then the portion of the original bud beyond this anal segment regressed until it was only a small ball of pigmented material. This ball then dropped off, leaving a growing, incomplete tail bud in place of the original head. When the implant included a well-developed head of the type produced after 5 to 9 days, no anal segment elements appeared. In some cases the bud continued to grow and organize, in others it regressed somewhat.

A young head bud, therefore, was not able to maintain itself against the more powerful tail-forming influence of the host's posterior segments, although this bud did modify the induced bud to some extent. Older buds, however, were retained without the host producing any additional structures.

Chaos nobilis Penard in permanent culture. A. A. Schaeffer.

From all published accounts of amoebas probably belonging to the genus *Chaos* Linnaeus, six well-attested species emerge. Three of these: *diffluens, nitida, neos, lescheri,* are uninucleate and two: *chaos* and *nobilis* are multinucleate. Excepting *nobilis,* all these species are readily cultured. *Nobilis* has been reported in the past (Vonwillier, Penard) as dying out in cultures. A few *nobilis* found in a ditch near Willow Grove, Pennsylvania, on April 20, 1941, maintained themselves in laboratory culture until now (August 20) and are slowly increasing in number. The rate of multiplication is much slower than in the other five species, for from 4 to 10 days elapse between divisions. This slow rate may be due to improper culturing methods, although from their appearance, one would judge these amoebas to be normal in every respect.

Von Stein (1867) was probably the first to see a *nobilis.* Next Butschli (1876) studied it and counted and measured nuclei in a wild culture. Penard studied it in 1902 and regarded it as a distinct species. Vonwillier later inclined to the view that the multinucleate amoebas which he found were like those of Calkins and Penard, but not like those of Butschli, Schubotz and Gruber. Lucy Carter described a multinucleate amoeba similar to Butschli's. The Willow Grove amoeba is similar to Penard's amoeba, but does not show the variability in number and nuclear size of Butschli's amoeba.

The two multinucleate species, *chaos* and *nobilis,* are distinctly different in size range, in nuclear structure, size, and number, and in rate of reproduction. Thus, the largest *nobilis* are about 250 μ in diameter and have from 80 to 90 ovoidal nuclei measuring 16 $\mu \times 12\,\mu$; the smallest are from 100 μ to 115 μ in diameter with 10 to 15 nuclei of about the same size and shape as those of the larger amoebas. The Willow Grove amoeba therefore corresponds very closely with Penard's *nobilis,* with Lucy Carter's, and with Butschli's multinucleate amoeba, but differs in nuclear number from Vonwillier's.

Further studies on Mactra egg cells. Victor Schechter.

The study of the problem of longevity in unfertilized *Mactra* egg cells was continued this summer along two directions. It was found that a non-dialyzable factor detrimental to the life of the eggs gradually develops in sea water considerably before the eggs show structural or functional deterioration. With regard to beneficial factors, dextrose down to the surprisingly low concentration of 0.001 per cent was found to be effective.

*The effect of centrifugation upon the oxygen consumption of Arbacia eggs.** Sidney F. Velick.

When unfertilized eggs of *Arbacia punctulata* are centrifuged in the cushioned medium of E. B. Harvey at a speed sufficient to stratify the cellular elements and stretch the cells to an axial ratio of about 1.5 to 1, the oxygen consumption is increased 60 to 120 per cent over that of the unstratified controls from the same egg suspension. The increment usually persists for several hours in the respirometer and does not result in membrane formation or cleavage. Upon fertilization, the oxygen consumption of the stratified eggs increases by the same order of magnitude as do the unstratified eggs. Return of the unfertilized stratified eggs to the spherical form is not accompanied by a decline in respiration, but a decline to the original level does occur after the spontaneous redispersion of the stratified granules has proceeded to a sufficient extent in a stationary vessel. The fat globules remain aggregated in a cluster long after the decline has occurred. As in the

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case of the unstratified egg, the oxygen consumption increases progressively upon too rapid shaking, an effect which has been observed by others on unstratified eggs and attributed to damage of the membrane. These experiments, undertaken at the suggestion of Dr. Kurt G. Stern, are being continued.

Ectodermization of the larva of Arbacia. Allyn Waterman.

A reinvestigation of previous work on *Arbacia* development has been attempted by exposing cleavage stages, swimming blastulae and early gastrulae to 2,4-dinitrophenol, 3,5-dinitro-o-cresol, pyocyanin, methylene blue, neutralized iodoacetic acid and to modifications of salt proportions in an artificial sea water formula which supported typical development through the pluteus stage. The reactions of these various stages differ little and then only in degree which is apparently correlated with the stage of development. Strong concentrations of respiratory affectors retarded or inhibited differentiation and gastrulation. Among the different abnormal types were found ectodermized embryos and undifferentiated exogastrulae. Weak concentrations accelerated development by several hours, without any differential effect upon the germ layer derivatives, and these larvae died earlier than the controls or abnormal types. Iodoacetic acid provoked ectodermization, indicating a shift of the control of development to the material at the animal pole. Omission of SO_4, $MgCl_2$, $CaCl_2$, or an excess of $MgSO_4$ in the artificial medium caused ectodermization. In all a great variety of abnormalities occurred which differed little from those provoked by many other methods. Undifferentiated exogastrulae cannot be considered a type of endodermization. During early development, at least, *Arbacia* embryos show a wide tolerance to most ionic variations in their environment.

The degree of ectodermization was variable within the same culture, showing a differential susceptibility between individuals. Completely ectodermized individuals often attained giant proportions, developed cilia and an exaggerated apical tuft, and survived as long as other types. Less completely ectodermized larvae often possessed some skeletal material and an undifferentiated gut.

While these results may be explained by the ectodermal-endodermal gradient hypothesis of Runnström, they appear to furnish no support to the suggestion of Lindahl that carbohydrate metabolism predominates at the animal pole (Needham and Needham).

Studies on Zoöchlorella-free Paramecium bursaria. Ralph Wichterman.

Isolation cultures were made of 40 specimens of *P. bursaria.* One strain disclosed *P. bursaria* to be completely free of the alga *Zoöchlorella* but to have instead great numbers of optically active crystals. These crystals were especially abundant in the posterior region of the ciliates. Unlike green *P. bursaria,* which generally settle to the bottom of the culture dish and congregate toward the strongest source of light, the "white" ones swim actively throughout the culture medium which consisted of desiccated lettuce infusion. White ones showed the mating reaction with green individuals of certain other clones. The mean daily fission rate of green specimens which mated with the white ones was 1.1 divisions while white *P. bursaria* showed a faster division rate, namely 1.5.

Since green *P. bursaria* contained no crystals and zoöchlorella-free individuals contained many, green individuals were kept in complete darkness for varying periods of time up to 25 days in order to find out whether the zoöchlorellae disappeared and crystals appeared. It was found that green individuals kept in darkness even for 25 days lost some but not all of their zoöchlorellae. On the other hand, optically active crystals appeared in such specimens and darkness did not prevent the mating reaction from taking place. However, large clumps of individuals, so characteristic in early stages of the mating reaction in the same

strains when subjected to light, did not form when they were kept in darkness for a considerable length of time.

Zoöchlorella suspensions from green individuals of the mating type opposite from the white *P. bursaria* were made. When a white specimen was placed in such a suspension of zoöchlorellae, the ciliate ingested the algae rapidly. Food vacuoles contained from one to five zoöchlorellae. However, in less than a day, the white *P. bursaria* became darkly granular, sluggish, then died. Yet when zoöchlorellae from another strain which also mated with the white ones were placed with them, no such lethal effect took place. Both of the above-mentioned clones of green *P. bursaria* showed the mating reaction with each other.

An experimental study of intracellular pH in the Arbacia egg. Floyd J. Wiercinski.

The problem of determining the exact pH of the living cell by the microinjection of indicators presents numerous difficulties. In addition to the salt and protein errors of the indicators these difficulties include membrane formation, granular breakdown, and most important of all, the uptake of indicators by the granules. In order to avoid factors due to the presence of granules, indicators were injected into the hyaline region of the centrifuged *Arbacia* egg.

Ten sulfonephthalein indicators and certain mixed indicators were used. A mixed indicator has the advantage of a sharp color transformation point at a given pH. Varied results were obtained with the indicators under different conditions of experimentation.

Tests with phenol red show a pH < 7.0, brom thymol blue < 6.8 and > 6.0, brom cresol purple + brom thymol blue < 6.6 and > 6.0, brom cresol purple < 6.8 and > 6.0, brom cresol green + chlor phenol red > 6.2 and < 5.8, chlor phenol red < 6.6 and > 6.0, chlor phenol red + aniline blue > 5.8, brom cresol green + Na Alizarine S > 5.8, methyl red > 5.8, and brom cresol green > 5.6. When eggs were immersed in 0.29 M $CaCl_2$ at pH 6.1 somewhat lower values were obtained. This may be due to an augmentation of the injury reaction.

The results indicate a pH somewhere in the neighborhood of 6.2 for the hyaline protoplasm of the *Arbacia* egg.

Heat produced by respiring whole blood of Tautoga onitis and Mustelus canis. E. Alfred Wolf, Maryon Dytche, John D. O'Neal and Milton Schaffel.

The colorimeter vessel was a triple-walled, silver-mirrored Dewar flask of about 100-cc. capacity. Temperature rise was measured with a Beckmann thermometer. Oxygen was supplied by bubbling. A method was found to prevent foaming which did not injure the cells. The fish used for the investigation were selected for their availability in Woods Hole waters. The constant temperature water bath was kept at 15.3° C.

The gas used for oxygen supply for *Tautoga* was oxygen and oxygen plus 5 per cent carbon dioxide; for *Mustelus*, because of lack of time, oxygen only was used. This high percentage of carbon dioxide was selected in order to have a basis of comparison for further work with higher vertebrates. The fish used in this investigation are normally not exposed to such high pressures of carbon dioxide and could not survive such conditions. At such pressures the blood of these fish could not be saturated with oxygen and the fish would suffocate in spite of the rich supply of oxygen (R. W. Root, E. A. Wolf and others). Our present work strikingly verifies these findings: in pure oxygen the rate of heat production remained constant for hours; in oxygen plus 5 per cent carbon dioxide this rate decreased after about one hour of exposure; all curves changed from straight lines

to curves of decreasing slope. We interpret this change as signs of approaching suffocation in an abundance of oxygen.

The rate of heat production of blood is of significant magnitude in the bioeconomics of fish. This rate in oxygen was 82 calories per kg. of blood per hour for *Tautoga* and 51 cal. for *Mustelus*. In oxygen plus 5 per cent carbon dioxide the rate decreased to 40 cal. for *Tautoga*. A fish of comparable size at 15° C. would produce about 360 cal. per kg. of body weight per hour.

Effect of differences between stages of donor and host upon induction of auditory vesicle from foreign ectoderm in the salamander embryo. C. L. Yntema.

Operations were performed on the embryo of *Amblystoma punctatum*. Foreign ectoderm from embryos at stages from 9 (early gastrula) to 28 (late head-process) was placed in the ear region of host embryos at stages from 12 (late gastrula) to 35 (onset of circulation). Prospective body ectoderm was used in experiments in which the donor was an early or middle gastrula. At older stages prospective gill ectoderm was transplanted. The animals were preserved at stage 46 (beginning of feeding). The contribution of the grafts was determined by retaining the Nile-blue sulfate stain of the grafts in sections.

The ability to induce a small vesicle was retained by a host as advanced as stage 35. The ability to induce was greatest, as measured by older grafts, during the neural groove stage (13) and during completion of neuralation (stages 19 and 20).

The response of the prospective body ectoderm from early and middle gastrulae was masked by induction of neural tissue from the grafts. Prospective gill ectoderm from stages 12 and 13 formed auditory vesicles if the hosts were neurulae. When older hosts were used, this potency was not realized. The most normal vesicles induced by hosts at stage 35 were from ectoderm taken from donors at stage 22 (early head process). Ectoderm from stage 28 formed small vesicles when placed in the ear region of stage 20.

The following are some general conclusions. If an embryo can no longer regenerate a labyrinth, its gill ectoderm possesses under certain conditions the capacity to form an auditory vesicle, and the ear region may still induce a vesicle. Ectoderm which is competent to form an auditory vesicle in the ear region of some stages is not competent to do so at certain other stages. Both the stage of the foreign ectoderm and the stage of the host are factors which determine the response to form a vesicle.